

CLINICOPATHOLOGICAL
AND EXPERIMENTAL STUDIES
OF THE VARIOUS FORMS
OF LEISHMANIASIS
FROM THE SUDAN AND
SAUDI ARABIA

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Malmö 1986

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The present thesis is based upon the results presented in the following publications, which will be referred to by their Roman numerals:

- I Morphological observations on visceral leishmaniasis in the Sudan.
Veress B, Malik MOA, Satir A, El Hassan AM.
Trop geogr Med 1974;26:198-203.
- II Morphology of the spleen and lymph nodes in fatal visceral leishmaniasis.
Veress B, Omer A, Satir A, El Hassan AM.
Immunology 1977;33:605-610.
- III Immunohistological investigations in chronic cutaneous leishmaniasis in Saudi Arabia.
Veress B, El Hassan AM, Kutty KK, Al-Gindan Y, Kubba R, Omer AHS.
Trop geogr Med (accepted for publication).
- IV Immunohistochemical demonstration of S-100 protein antigen-containing cells in chronic cutaneous leishmaniasis.
Veress B, El Hassan AM.
Acta path microbiol Scand Sect A 1985;93:331-334
- V Vascular changes in human leishmaniasis.
Veress B, El Hassan AM.
Ann Trop Med Parasitol (accepted for publication).
- VI The ultrastructural morphology of human cutaneous leishmaniasis of low parasite load.
El Hassan AM, Veress B, Kutty MK.
Acta Derm Venereol (Stockh) 1984;64:501-505.
- VII Electron microscope investigations on leishmaniasis in the Sudan. I. Morphometric studies on *Leishmania* amastigotes in various forms of human leishmaniasis.
Veress B, Abdalla RE, El Hassan AM.
Ann Trop Med Parasitol 1980;74:421-426.
- VIII Electron microscope investigations on leishmaniasis in the Sudan. II. Ultrastructural morphology of macrophage-parasite interaction in human and hamster macrophages *in vivo*.
Veress B, Abdalla RE, El Hassan AM.
Ann Trop Med Parasitol 1981;75:607-613.

- IX Visceral spreading, depletion of thymus-dependent regions, and amyloidosis in mice and hamsters infected intradermally with *Leishmania* isolated from Sudanese cutaneous leishmaniasis.

Veress B, Abdalla RE, El Hassan AM.

Brit J exp Pathol 1983;64:505-514.

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INTRODUCTION

The Democratic Republic of the Sudan is Africa's largest, but most scantily populated, country (area 2,505.813 square km; population: 18,806.000; capital: Khartoum). Its vastness encompasses deserts in the North, mountains in the West and East, and savannas and a gigantic swamp in the South. The people can be divided into two large groups, Arabs in the North and Negroid-Africans in the South. The name of the country comes from the medieval Arabic term "Belad as Sudan" meaning "Land of the Blacks" (1).

Leishmaniasis is an infectious protozoal disease caused by different types of the unicellular flagellate *Leishmania* (Phylum: Protozoa, Order: Kinetoplastida, Genera: Trypanosomatida). During their life cycle the protozoons have a flagellar and an aflagellar form. The flagellar promastigotes live in the gut of the vector *Phlebotomus* sandfly. The aflagellar amastigotes are obligate intracellular parasites living in the histiocytes of the vertebrate host (2).

Leishmaniasis is a disease of the tropical and subtropical regions of the earth, affecting well over 10 million people (3). Of the diseases caused by protozoa it is considered second in importance only to malaria (4). Marsden (5) wrote in 1979 that "the knowledge on leishmaniasis stands today where Chagas' disease stood 15 years ago and malaria at the end of World War II". Its distribution is mainly that of the vector sandfly. The disease is generally regarded as a zoonosis, carnivores and rodents serving as the non-human host (6). It can also be seen in Northern- and Western-Europe (7-17) due to modern travel which takes thousands of tourists and businessmen each year to the Mediterranean basin or Africa, where the disease is endemic (18). A few cases of visceral leishmaniasis (VL) have also been described in Sweden (19-21) and since 1977 I have also had the opportunity of examining sections from cutaneous leishmaniasis (CL) cases found in this country.

In the Old World human leishmaniasis occurs in two forms depending on the clinical symptoms and the affected organs:

- | | |
|-----------------------|---|
| (a) Tegumentary forms | Cutaneous leishmaniasis
chronic lupoid leishmaniasis
oriental sore
diffuse cutaneous leishmaniasis
Mucocutaneous leishmaniasis
Mucosal leishmaniasis |
|-----------------------|---|

(b) Visceral forms	Visceral leishmaniasis or kala-azar
	Mediterranean kala-azar
	East-African kala-azar
	Indian kala-azar

Leishmaniasis, like leprosy, can be regarded as a spectral disease, with the self-healing oriental sore at one end of the spectrum and the almost always fatal kala-azar at the other (22). Nevertheless, within the cutaneous and visceral groups intermediate forms can also be recognized both clinically and morphologically (23-26).

The various clinical forms of the disease were thought to be caused by different species of *Leishmania*. It was generally accepted that *L. donovani*, *L. infantum*, and *L. chagasi* caused the visceral disease (Indian + East African, Mediterranean, and South American, respectively) that *L. braziliensis braziliensis* was the major cause of espundia, while CL was caused by *L. tropica*, *L. major*, *L. aethiopica*, members of the group *L. mexicana*, and some species of the group *L. braziliensis* (6,27-29). The experimental investigations of the past few years have shown, however, that the genetic control of the host plays a very important, if not decisive, role in the course and the development of the various forms of the disease (30-34).

When we started our investigations on leishmaniasis the Sudan seemed to offer unique possibilities. First of all, leishmaniasis occurs in three different forms in this country: cutaneous, pure mucosal (ML), and visceral (35-50). Of these forms, ML has not been reported outside Africa and, indeed, only one case, a case from Morocco (51), has ever been described from outside the Sudan. Secondly, earlier taxonomic investigations had been able to isolate only one *Leishmania* species in the Sudan, namely *L. donovani* (52). In parenthesis, it should be noted that recent results point to the occurrence of *L. major* there also (53,54). Bray (29) stated in 1974 that one of the most important taxonomic problems was "whether all strains recovered from man, animals, and sandflies in the Sudan are identical". Thirdly, detailed histomorphological analysis of the various leishmaniasis forms had not been performed until the last decade and the published papers on Sudanese leishmaniasis (about 50) had dealt with it mainly from the clinical and epidemiological viewpoints. These were the facts that led us to study the histomorphology of human leishmaniasis in the Sudan, and, later on, of CL in Saudi Arabia and of experimental leishmaniasis in mice and hamsters. The results of these studies are summarized in this thesis.

THE AIMS OF THE STUDIES

1. To study the histological changes of Sudanese kala-azar, with special regard to the changes in the lymphoid organs, and the accompanying secondary alterations in the other parenchymatous organs.
2. To analyse the inflammatory response in ML and CL and to study the immunohistology of chronic CL.
3. To determine the type of the *Leishmania* in the Sudan by applying ultrastructural morphometry.
4. To study the ultrastructural morphology of the macrophage-parasite relationship and to compare the findings in human and experimental material.
5. To follow the morphological changes in experimental infection, with special regard to the type of the inflammatory response, the alterations of the lymphoid organs, and the possible development of "secondary" leishmaniasis-related disease.

MATERIAL AND METHODS

The details of the human and experimental material, the isolation of the parasites, the morphometric and statistical methods, and the staining procedures for light microscopy, immunohistochemistry, polarization microscopy and electron microscopy are described in papers I - IX.

RESULTS

OBSERVATIONS ON THE HUMAN MATERIAL

Morphological alterations of the various organs in kala-azar (I, II and V)

The geographic distribution of the disease in the Sudan is shown in Fig. 1 and the main clinical symptoms are shown in Fig. 2 and Table 1. The main causes of death were bacterial (15 cases) or mycotic (2 cases) infections of the lungs, suppurative pyelonephritis (2 cases), pulmonary haemorrhages (7 cases), and uraemia secondary to the nephrotic syndrome (2 cases).

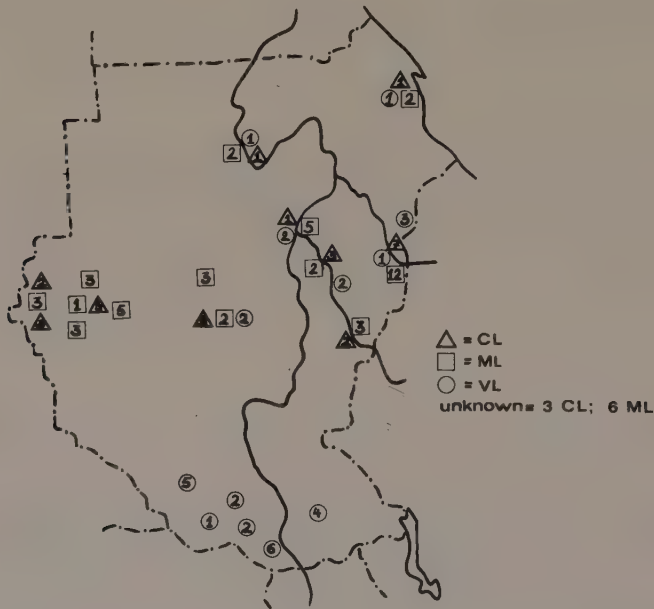


Fig. 1. The map of the Sudan showing the distribution of human CL, ML, and VL

Signs	Fever	Anaemia	Leukopenia	Haemorrh. diathesis	Hepato-megaly	Spleno-megaly
N ^o of cases	26	29	15	10	22	27

Table 1. The leading clinical features of kala-azar.



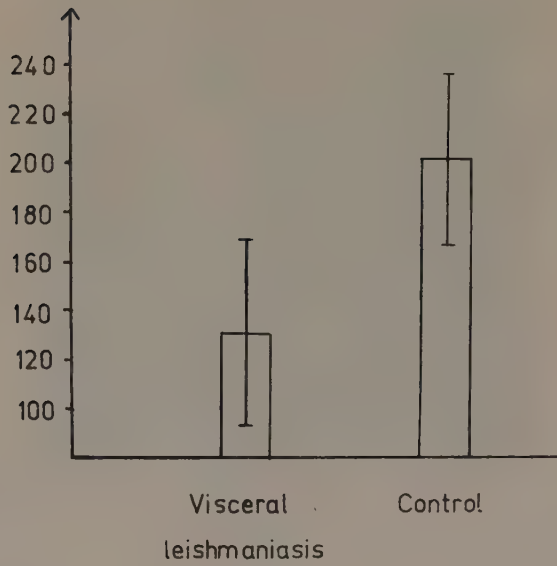
Fig. 2. VL with hepato-splenomegaly (14 years old boy).

The main histological findings were:

1. A significant reduction ($p < 0.02$) in the size of the Malpighian corpuscles as compared with that of the control group (Fig. 3).
2. Necrosis of the small lymphocytes around the central arteries in the white pulp.
3. The reduction in number of the small lymphocytes in the paracortical areas of the lymph nodes.
4. The accumulation of hyaline in the non-necrotized Malpighian corpuscles and the primary follicles of the lymph nodes.
5. The presence of large PAS⁺, PTAH⁺ droplets in the cytoplasm of the "littoral" macrophages of the splenic sinuses (Fig. 4).
6. The occurrence of erythrophagocytosis in the spleen.
7. Systemic vascular alterations consisting of subendothelial oedema, hyalinosis, and intimal proliferations.



Mean diameter of the
Malpighian's corpuscles



THE RATIO OF THE WHITE PULP TO $1 \times 10^6 \mu\text{m}^2$ SURFACE
OF THE SPLEEN

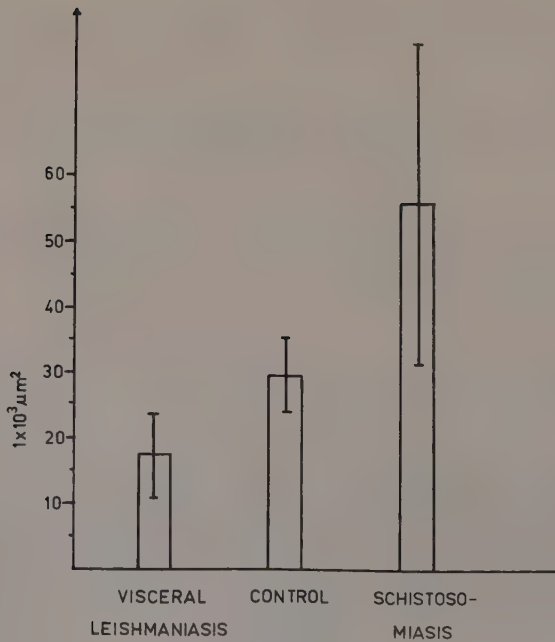


Fig. 3. The size of the white pulp area in the fatal cases of VL.

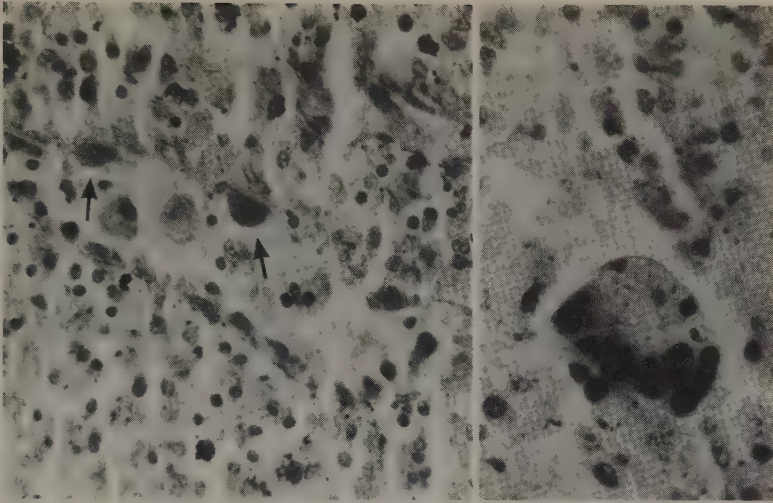


Fig. 4. A: VL, case 99/69. PAS positive droplets in the cytoplasm of the "littoral" macrophages lining the sinus (arrows). PAS, x240.

B: VL, case 23/61. Several PTAH positive droplets in the cytoplasm of a large macrophage in the spleen. PTAH, x360.

Observations on Sudanese mucosal leishmaniasis (V, VII, VIII)

The geographical distribution of the disease is shown in Fig. 1. Of the 52 patients, 46 were males. The average age was 43 years. The duration of the illness varied from 5 months to 10 years. None of the patients mentioned previous cutaneous lesions and very thorough investigations did not reveal any scarring or other signs of CL.

The main symptoms were pain and bleeding. Breathing through the nose was very difficult and the sense of smell severely affected in several cases. If the disease involved the larynx, hoarseness developed. The regional distribution of the mucosal involvement is shown in Table 2.

Region	Nose	Lip	Palate	Cheek	Tongue	Larynx	Nose Palate	Nose Pharynx
N ^o of cases	16	10	11	1	3	5	1	3

Table 2. The regional distribution of ML.

Gross appearance of ML. Two forms could be differentiated: (a) the nodular form, in which firm nodules of various sizes were seen (Fig. 5), (b) the diffuse form, which very often caused ulceration.

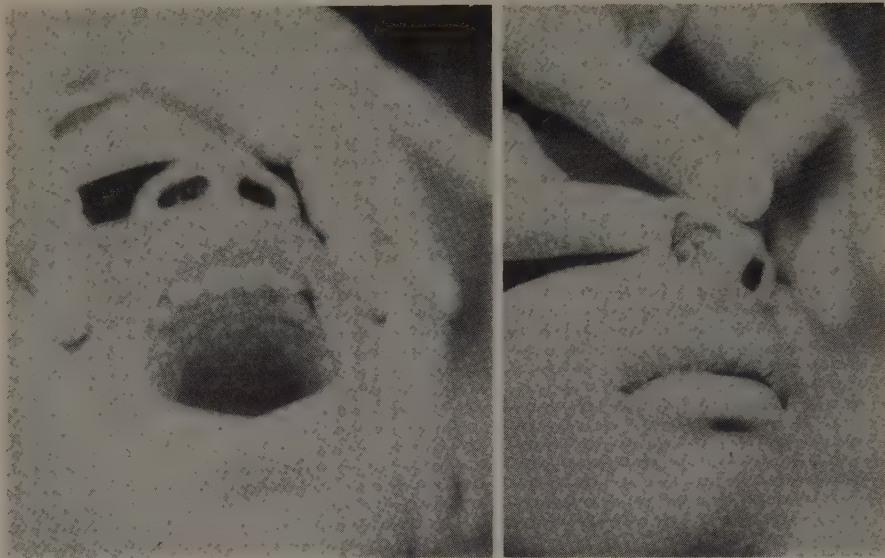


Fig. 5. ML showing thickening of the mucosa of the hard palate (A) and that of the nasal mucosa (B). Male, 36 years old.

Histologically, the inflammatory reaction showed four basic patterns without any definite relation to the gross appearance of the disease.

1. In 4 cases the mucosa was diffusely infiltrated by large, pale, parasitized histiocytes (Fig. 6). Intermingled with the histiocytes some plasma cells and very scanty lymphocytes could be found. Neither activated macrophages nor epithelioid cells were seen.
2. In 9 cases the inflammation showed a roughly equal mixture of lymphocytes, plasma cells, and mainly activated macrophages. The numbers of parasite-laden histiocytes were invariably low and a striking feature was the occurrence of epithelioid cell granulomata (Fig. 7).
3. In 21 cases small lymphocytes and proliferating capillaries with hypertrophic endothelial cells were the dominant cellular components (Fig. 8). Only very few parasitized histiocytes could be observed but activated macrophages were always present sometimes even organized granulomata composed of activated histiocytes could be found. Plasma cells were also present but they were in the minority.

4. In 16 cases a predominance of plasma cells was found but there were also a relatively large number of sometimes parasite-laden histiocytes (Fig. 9).

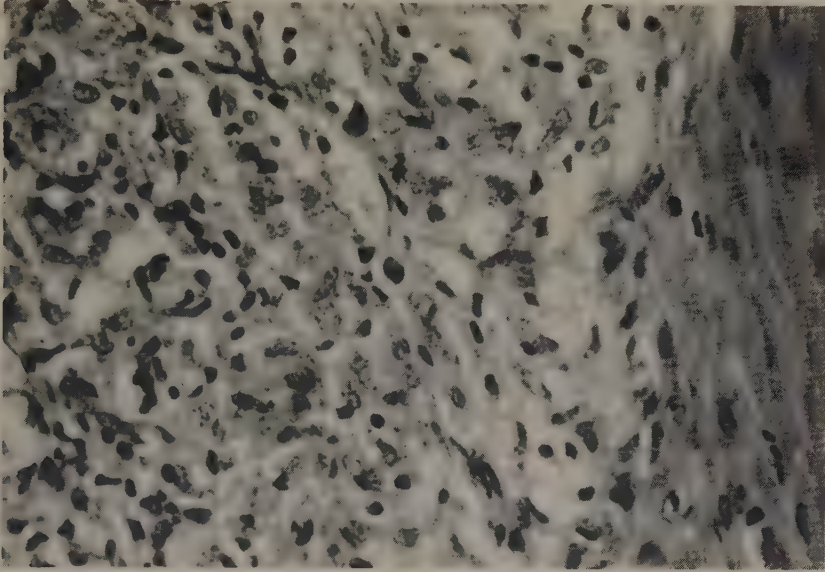


Fig. 6. ML, case D/5647. LyD type of the response with numerous parasitized macrophages and a few plasma cells. H & E, x220.

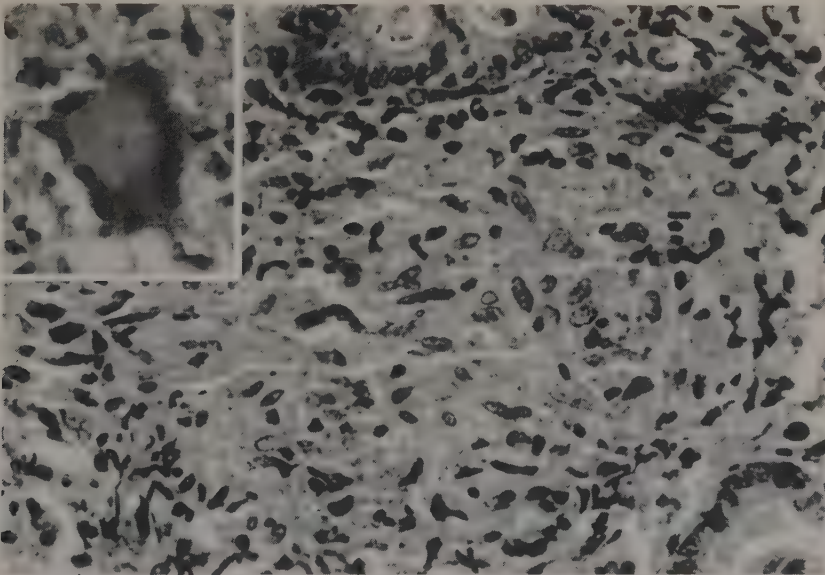


Fig. 7. ML, case D/9186. Mixed type of response with granuloma (A) and Langhans giant cell (B). H & E, x180.

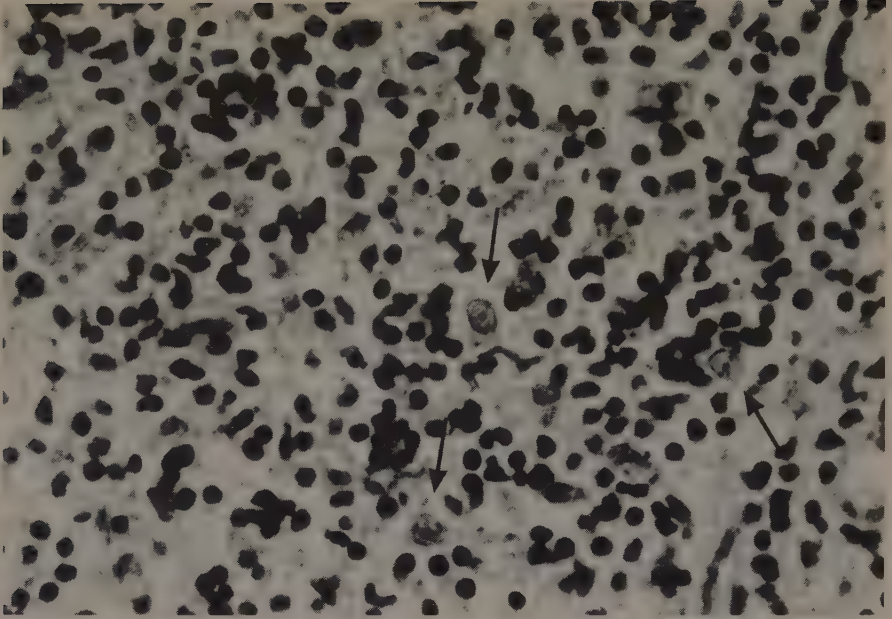


Fig. 8. ML, case C/7399. LyP type of response with numerous small lymphocytes and a few macrophages (arrows). H & E, x220.

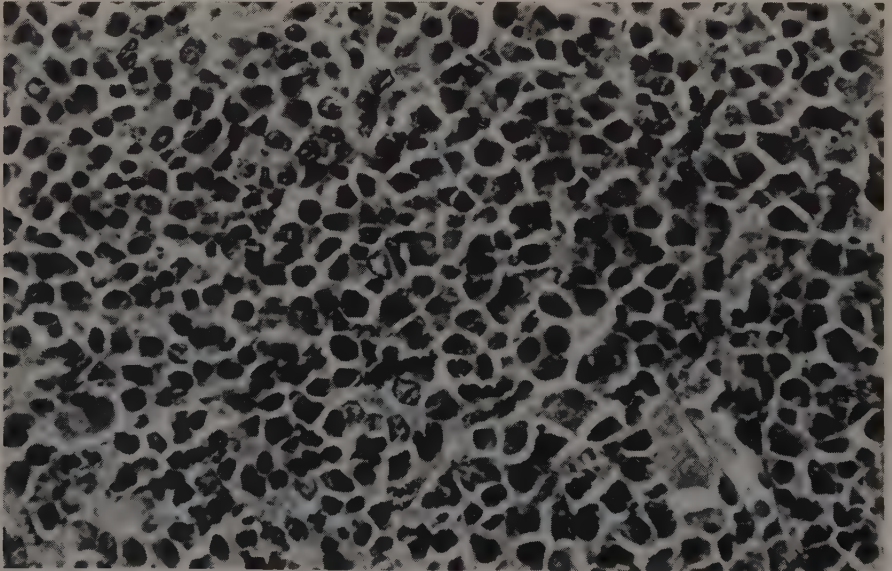


Fig. 9. ML, case A/8852. PIP type of response with many plasma cells. H & E, x220.

In two cases repeated biopsies during treatment showed transformation of the inflammatory reaction from the first type to the third one in parallel with healing.

The vascular lesions consisted of fibrinoid necrosis, hyalinosis, intimal proliferation, and vasculitis. In six cases the development of fibrin thrombi was found.

Observations on Sudanese cutaneous leishmaniasis (this thesis)

The geographical distribution of the cases is shown in Fig. 1. The average age was 33 years. Of the 37 patients, 33 were males. Preceding VL was reported in only two cases.

Clinically, the lack of pain and itching was typical. Regional lymphadenopathy could be found in five cases. The lesions usually healed, but in a few cases the disease became chronic and almost therapy-resistant.

Gross appearance of CL. (a) The nodulo-ulcerating form was observed in 22 patients. There was a very firm, 1-5 cm large nodule or numerous small nodules with superficial ulceration in seven of the 22 cases. (b) The ulcerating form was found in 14 patients. One or more very deep ulcers developed from the very beginning (Fig. 10). The ulcers were rounded with very sharp, firm edges.

In one patient two lesions developed, one on the chin and another on the forehead, showing diffuse thickening of the skin without ulceration.



Fig. 10. CL showing two large ulcers with sharp, firm edges on the hand of a 42 years old man.

Histologically, the inflammatory response was similar to that of ML. Most of the cases showed the predominance of lymphocytes (21 cases). Plasma cell preponderance was seen in 5 cases, and a mixed cellular composition was found in 6 cases. Large, pale histiocytes were observed in fairly large numbers in four cases, but the numbers of parasitized histiocytes were low even in this type of inflammation. They were, however, numerous in the diffuse cutaneous lesion of one patient.

Observations on CL from Saudi Arabia (III, IV, V, VI)

The disease showed four histological patterns, similar to those of Sudanese ML and CL. Electron microscopy revealed the occurrence of apoptosis of single macrophages and the presence of epithelioid cells. Immunohistology showed IgG- and IgA-positivity in the cytoplasm of some of the macrophages and squamous epithelial cells, as well as in the endothelial cells and around the diseased vessels. There was a marked difference regarding the lysozyme content between the various cells of the mononuclear phagocytic system: activated macrophages showed very strong and diffuse cytoplasmic lys⁺; epithelioid cells and giant cells were either lys⁻ or contained only a few weakly stained lys⁺ granules. S-100⁺ lys⁻ cells, the members of the dendritic cell system, could also be found scattered in the infiltrate.

ULTRASTRUCTURAL-MORPHOMETRIC STUDIES (V).

The diameters of the protozoons from Sudanese VL, ML, and CL did not differ significantly from each other. Neither did the contour lengths and subpellicular tubule-numbers. The values of these measurements corresponded to those described in *L. donovani* (Table 3). It is of interest that transversally cut subpellicular tubules could be found in only 25-35% of the total circumference of the parasites.

Microtubule count of the various Leishmania parasites

<i>L. enriettii</i>	120 - 220	Kenan, 1975
<i>L. mexicana</i>	130 - 220	Garnham & Bird, 1962
	<u>114 - 118</u>	Creemers & Jandin, 1967
		Gardener <u>et al.</u> , 1977
<i>L. m. amazon.</i>	125	Gardener, 1974
<i>L. tropica</i>	80 - 125	Angelopoulos, 1970
	90 - 94	Hentzer & Kobayashi, 1977
<i>L. donovani</i>	80 - 120	Sanyal & Sen Gupta, 1967
	<u>77 - 81</u>	Gardener <u>et al.</u> , 1977

Table 3. Summarizing the data of the literature on the numbers of SPT in the various *Leishmania* species.

ULTRASTRUCTURAL MORPHOLOGY OF MACROPHAGE-PARASITE INTERACTION IN HUMAN AND HAMSTER MACROPHAGES IN VIVO (VIII)

The majority of the macrophages from human ML showed signs of active cellular metabolism and the parasites, if they were found in these cells, were tightly surrounded by "host membrane". The heavily parasitized hamster macrophages showed signs of cellular degeneration and the parasites were found in large parasitophorous vacuoles.

THE MORPHOLOGY OF THE LESIONS IN MICE AND HAMSTERS EXPERIMENTALLY INFECTED WITH AMASTIGOTES FROM HUMAN CUTANEOUS LEISHMANIASIS (IX)

In all the mice and in the majority of the hamsters VL developed. It was characterized by the predominance of heavily parasitized, non-activated macrophages, a selective T-cell depletion in the spleen and lymph nodes, and the development of a reactive, secondary amyloidosis.

In some of the hamsters the inflammatory infiltrate was of the mixed cellularity type, with very scanty parasites, numerous activated macrophages, and lymphocytes and plasma cells. Erythrophagocytosis by splenic macrophages was observed by the electron microscope and an intimate contact between lymphocytes and macrophages was also seen (Fig. 11).

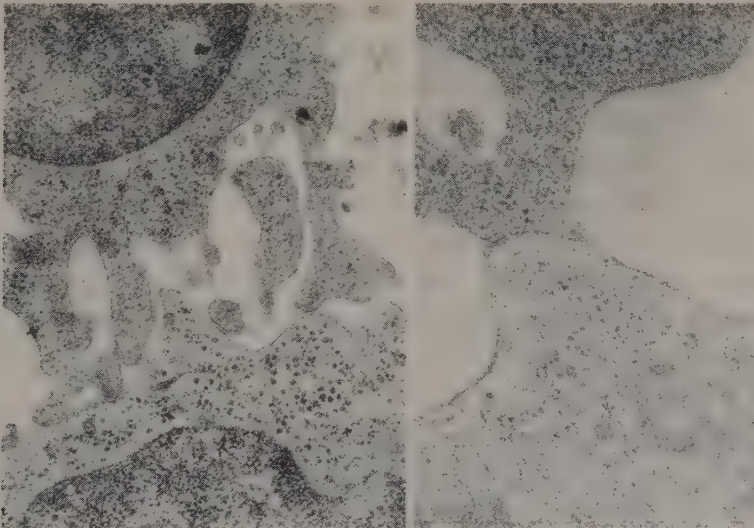


Fig. 11. Hamster, spleen, 18 weeks after inoculation of amastigotes from a case of CL. A: close contact between a lymphocyte and a macrophage with the help of short cell-projection. Ua-Lc, x22 000. B: showing the detail of a contact between a lymphocyte and a macrophage. Ua-Lc, x 38 000.

DISCUSSION

THE TYPE OF THE LEISHMANIA SPECIES IN THE SUDAN

In the last decade there have occurred fundamental changes in the methodology of the species-determination of *Leishmania*. Formerly, the geographical distribution, the clinical picture, the tissue-tropism, and the parasites' simple morphological parameters were the basis for the classification (6,29). The use of more exact and specific methods started in 1966, when Adler and his co-workers described differences in the antigenic properties of the various *Leishmania* species and used these properties to identify various strains (52). In the past 10 years serological (55-58) and biochemical (59-66) methods, and more recently monoclonal antibodies (67) have been introduced and used successfully in species-determination. Ultrastructural differences have also been noted between the various species, e.g. by the use of the electron microscope a variation in the number of subpellicular tubules has been discovered (68-73) (Table 3). Not only the number of these tubules, but also their pattern of arrangement can give some help in the typing (74). Gardener et al. (73) found tighter and looser spiralling of the tubules in various species.

The situation regarding the type of *Leishmania* causing VL, ML, and CL in the Sudan is far from clear, as has already been pointed out (page 8). Clinical observations on the occurrence of ML and VL in the same patient (Kirk (42) and one of our cases) and the investigations of Adler et al. (52) and Schnur and Zuckerman (58) point toward the possible existence of one common species in this country. Recently, Chance et al. (53) showed that 15 strains isolated in the Sudan were identical with *L. donovani* S. 1. However, the features of one Sudanese strain isolated from a case of CL from Darfur province corresponded biochemically to *L. major*, but serologically it was identical with *L. donovani*. Abdalla (54) reported also the occurrence of *L. major* in several CL cases in the Sudan and put forward the theory that VL and ML are caused by *L. donovani*, whereas *L. major* was the common cause of CL. He did not exclude the possibility that *L. donovani* was the cause of some of the cutaneous cases.

The results of our statistical analysis of the subpellicular tubule count showed no significant differences between the amastigotes found in the three clinical forms of leishmaniasis in the Sudan. The values conform closely to those found for *L. donovani* (73). Nevertheless, the amastigotes in our material had both transversally and longitudinally cut tubules, pointing to a tighter helix than that of *L. donovani* (73). The amastigotes examined in our morphometric analysis originated from CL cases which came from regions of the country other than Darfur, (namely from Kassala and Khartoum provinces), and therefore our results do not necessarily contradict

those of Chance et al. (55) and Abdalla (54). Our observations seem to suggest that

- (1) the parasites found in our cases of Sudanese VL, ML, and CL form a subgroup of *L. donovani*,

a similar theory was put forward by Manson-Bahr (75), who, noting the difference between Indian and East-African kala-azar, raised the possibility that a special type of *L. donovani* is the causative parasite in East Africa. Bray (29) was sympathetic to this theory and was ready to accept the recommendation of Garnham (6) of naming the parasite of Sudanese kala-azar as the subspecies *L. donovani archibaldi* ;

- (2) the same species of *Leishmania* can cause various clinical forms of leishmaniasis in humans.

THE CELLULAR COMPOSITION OF THE INFLAMMATORY RESPONSE IN CUTANEOUS AND MUCOSAL LEISHMANIASIS

The extensive morphological, immunological, and genetic research of the past 20 years has contributed much to our understanding of the pathogenesis of several tropical diseases. It is recognized, for example, that the various histological types of leprosy are the morphological expressions of the status and responsiveness of the immune system (76-78).

Heyneman (79) regarded the skin as an organ of primary importance in leishmaniasis because the skin is the place of the first encounter between the protozoons and the host's defence. He also stated that the immunity to VL resided in the skin. The generally accepted view today is that cell-mediated immunity is the main defence against the parasites (22,80,81) but there are some recent observations that suggest that the humoral immune response is also important, even if it acts during a shorter, well-defined period (82-85).

The genetic research of the past 10 years on various strains of inbred mice has revealed the importance of the host's genes on the outcome of the infection. These investigations also seem to clarify the role and relationship of the cellular and of humoral immunity in the defence against the parasites. It is now clear that the host's response during leishmaniasis can be divided into two phases: in the initial phase the innate natural resistance is responsible for the defence. In the case of *L. donovani* infection this is under the control of a single *Lsh* gene on chromosome 1 (86,87); whereas in *L. tropica* infection it is controlled by non-H-2 genes

with a minor H-2 gene influence (88,89). In the second, late phase, acquired T-cell mediated immunity is responsible for the protection. It has been shown that this is controlled by non-H-2, Ir-2 genes, with the help of the H-2 complex and the H-11 locus (90,91).

During studies on the regulation of the *Leishmania* population within the host, Bradley & Kirkley (92) found that the inbred strain NMRI showed a different histologic picture during the late resolving phase of the infection from that of the non-resolving outbred PO mice. The "cure" type, occurring in the NMRI strain, was characterized by lymphocytes and activated histiocytes by the 80th day after the infection. Epithelioid granulomata were also found. Parasitized histiocytes were rare or totally absent. The "noncure" type, occurring in PO mice, was dominated by heavily-parasitized, large, pale histiocytes and plasma cells, but no or very few lymphocytes were seen. The few granulomata were composed of parasite-loaded histiocytes.

Our histomorphological study on CL and ML, and the various descriptions in the literature dealing with the histology of human CL and mucocutaneous leishmaniasis, show that the histological picture is more complex in man than in mice. Bryceson (23) classified Ethiopian CL into 5 classes based upon the number and quality of macrophages and the tuberculoid granulomata. Eleven years later Ridley (93) suggested a new classification using two basic histological features, necrosis and cellular composition, as the cornerstones. He formed three geographical groups (Ethiopian, Afro-Asian, and Central-American), each having special features. Because this classification resulted in 12 types, it was difficult to use in every day practice.

Recently, Ridley & Ridley (94) have shown that the histological picture reflected 3 basic forms of response (intact macrophage, diffuse necrosis, and focalized necrosis) with the development of tuberculoid granulomata in each form. They pointed out that there was no single straight-line spectrum in CL, though a two way spectrum could be delineated: from anergic macrophage to tuberculoid granuloma and from intracellular digestion of the parasites to lysis of the parasitized macrophages.

We have found the inflammatory infiltrate to consist of cells of the mononuclear phagocytic system, lymphocytes, and plasma cells. Depending on the dominant cell type and the immunohistological pattern the cases of CL and ML can be divided into four groups: (a) non-activated, parasitized or vacuolized macrophages predominating, (b) the cellular composition is a roughly equal mixture of the three

cell types, (c) lymphocytes predominating, and (d) plasma cells predominating. Since experimental investigations had revealed the crucial role of the lymphocytes in activating the macrophages and making them able to kill the parasites (22,34,95) it seemed logical that the terminology should reflect their importance. Hence, we chose the terms: lymphocytic depletion, mixed cellularity, lymphocyte preponderance, and plasma cell preponderance, respectively.

A most remarkable feature of the cellular infiltrate was the reversed ratio between lymphocytes and parasite-loaded histiocytes. Similar observations were made in both human CL and mucocutaneous leishmaniasis (23,27,93,94,96-98), and in animal experiments (22,92,99,100). The fact that the lymphocytes were scanty and the non-activated macrophages were fully packed with amastigotes in the first group supports the following assumptions: (i) the macrophages are able to internalize the parasites but are unable to destroy them in the absence of the lymphocytes, as has also been suggested by others (22,101), (ii) the presence of lymphocytes in great numbers, and effective cooperation and information exchange between lymphocytes and macrophages are necessary to deal successfully with the parasites. The crucial elements of this cooperation are T-cell sensitization through antigen presentation by cells of the dendritic cell system (102-104) and activation of the macrophages by lymphokines of sensitized lymphocytes (22,105,106).

The four histological groups can represent the various phases of the disease and/or the responsiveness of the host. At the beginning, the lack of specific T-cell sensitization and macrophage activation makes it possible for the parasites to live and multiply undisturbed within the macrophages. Later on, the numbers of lymphocytes and plasma cells increase, and the morphological signs of macrophage activation can also be seen. The elimination of the parasites starts either within the activated macrophages or through the lysis of the whole cell, which is probably due to immunological necrosis. The development of epithelioid cell granulomata may indicate a hyperergic reaction. In certain individuals, however, the immunological defence is not effective and the disease becomes either a chronic, non-healing local process or can be transformed to a wide-spread, generalized disease, kala-azar.

The failure of defence is manifested by the absence of macrophage activation. This can be the result of excess antibody (107), T-lymphocyte's depletion (34), or the inadequate response of macrophages due to failure of genetic control (90). Our immunohistological findings and the vasculitis and intravascular coagulation in CL point to the presence of immune complexes due to an excess amount of antibodies locally. Antigen presenting S-100⁺ reticulum cells were found in our CL cases with

low parasite load, whereas, according to our preliminary, unpublished observations, these cells were absent in cases dominated by parasitized, non-activated macrophages. These observations support the hypothesis that T-cell sensitization is an important step in the defence process against *Leishmania*.

The immunohistological investigations on chronic CL cases revealed a marked difference in the lysozyme-positivity between activated macrophages, epithelioid cells, and giant cells. The activated macrophages always showed a strong reaction, while the epithelioid cells and giant cells were either negative or contained only weakly stained granules in the cytoplasm. Non-activated, parasitized macrophages were completely devoid of detectable lysozyme (unpublished observations), hence we believe that the lysozyme content may be the expression of the different level of cell activation. To the best of our knowledge, there is only one similar observation described in the literature and this was made on leprosy (108).

The presence of IgG^+ and IgA^+ squamous epithelial cells in the epidermis might suggest that the necrosis of the skin and the ulceration in CL are immunologically induced phenomena. This theory was put forward by Bryceson et al. (22), who found cell necrosis in the prickle-cell layer followed by ulceration. Monroy et al. (99) agreed with this theory suggesting that the skin ulceration was brought about by the same immunological mechanism that caused the destruction of the macrophages.

In summary:

- (1) Four histological groups of inflammatory infiltrate can be differentiated in both CL and ML: lymphocytic depletion, mixed cellularity, lymphocyte preponderance, and plasma cell preponderance.
- (2) The lymphocyte/parasitized histiocyte ratio is reversed in the inflammatory infiltrate and can be regarded as the morphological expression of the ability of the host to deal successfully with the parasites.
- (3) There is a marked difference in the detectable lysozyme content between the various cells of the mononuclear phagocytic system, the activated macrophages showing the strongest positivity, suggesting that the lysozyme content can serve as a marker of cell activation.
- (4) $\text{S-100}^+\text{lys}^-$ reticulum cells are present in the inflammatory infiltrate of chronic CL with low parasite load suggesting that antigen presentation takes place, and

- (5) apoptotic necrosis of single non-parasitized macrophages and groups of IgG⁺IgA⁺ squamous epithelial cells can be found in chronic CL, supporting the view that immunologically induced necrosis occurs in the disease.

THE CHANGES IN THE LYMPHOID ORGANS IN VISCERAL LEISHMANIASIS

The Sudanese VL is of the East African form, which differs from the Mediterranean and Indian forms not only in the vectors and reservoirs (28,29) but also in certain epidemiological and clinical aspects (27). Thus, whereas the Mediterranean form occurs mainly in children up to 10 years of age (11), and the Indian form has a peak incidence at 10-20 years (27), the East African form is common in adults (48,109,110). East African kala-azar shows the most malignant course, with more than 20% mortality, and spontaneous healing is far rarer than in the other two forms (28,111,112).

The clinical and epidemiological features in our Sudanese cases correspond well to these characteristics of East African kala-azar. The malignant course of kala-azar in the Sudan was demonstrated by 13 cases in our series who died in spite of treatment. The commonest causes of death were septicaemia, pulmonary haemorrhages, and nephrosis. From the literature it is also known that cancrum oris, a gangrene of the face similar to noma faciei, sometimes develops (109). These complications raise the suspicion that a severe defect of the immune system develops in fatal VL cases.

Manson (113) described an ancient custom among the local inhabitants living in leishmaniasis-endemic regions in East Africa of inoculating the children's skin with material obtained from the base of leishmaniasis ulcers. In Kenya immunity is said to last for at least 10 years following recovery from kala-azar (114). Active immunization with *L. adleri* was also carried out in East Africa (115,116). Other reports also lend support to the idea of the importance of the immune system in the outcome of VL. Hoogstraal et al. (117) described the case of one of their Sudanese laboratory technicians, who for reasons of economy did not eat sufficiently and who became the only member of the group to contract VL. The suppression of cell-mediated immunity, which is a known complication of malnutrition (118), was presumably a contributing factor in his case. Immunosuppression in a renal-transplant patient caused the development of fatal VL (119). Bacterial infections (*M. tuberculosis*, for instance) can also impair the immune response. Probably this is what happened in one of our patients, who at the time of presentation showed no signs of VL but had tuberculosis and ML. Later on, in spite of both anti-leishmanial and anti-tuberculous treatment, hepato-splenomegaly developed and she died. The autopsy and the

histology showed, besides tuberculosis of the lungs and an ML lesion of the nose, a very severe VL.

There have been observations on the morphological alterations of the immune system in kala-azar. Atrophy of the Malpighian corpuscles has been described both in human VL and in experimental animals (120, 121). Plasmacytosis and proliferation of the histiocytes in the red pulp of the spleen in human VL have also been noted (120,124,125). The observations were, however, of a qualitative nature and the authors did not attempt to carry out a morphometric analysis.

Our quantitative measurements showed a significant decrease of the white pulp area of the spleen in the fatal VL cases. This reduction meant that the size of the Malpighian corpuscles was only about a third of that found in normal spleens. As a contrast to VL, we found a marked enlargement of the Malpighian corpuscles (their size was nearly doubled, unpublished data) in another tropical disease, schistosomiasis. More recent experimental investigations have supported our findings by showing a significant fall in the percentage of T-cells in the spleen (32,122). A reversed T/B cell ratio has also been found in the peripheral blood of kala-azar patients (123).

One can argue that the 3-4-fold enlargement of the spleen in VL can compensate for this reduction of the size of the Malpighian corpuscles. The qualitative histological analysis provided evidence against this, by showing that the size-reduction was due to extensive necrosis of the small lymphocytes of the T-dependent region and that hyperplasia of the red pulp was responsible for the enlargement of the spleen. The plasmacytosis in the red pulp was, however, not effective in controlling the proliferation of the parasites, as demonstrated by the presence of numerous non-activated, parasite-loaded macrophages. This type of response is similar to the reversed lymphocyte/parasitized histiocyte ratio observed in CL and ML. Similar changes could also be found in our experiments (page 19).

These observations show that both in human and in experimental leishmaniasis a severe depletion of the thymus-dependent regions develops, accompanied by plasmacytosis. Turk (126) and Bryceon et al. (22) viewed human kala-azar as associated with suppression of the specific cell mediated immunity, leading to immune paralysis. Under these circumstances the body tries to defend itself with humoral immunity, but without success. This condition was called "immune deviation" or "split-tolerance" (76). It is also seen in leprosy (126), in cytomegalovirus (127),

and in *P. falciparum* (128) infections.

What is the mechanism behind the T-depletion in VL? There are at least two possibilities, such as the effect of an antilymphocytic antibody (129), or an overloading of the body by leishmanial antigen (130). Both of these are supported by experimental data. Paracortical depletion was produced in the lymph nodes of *L. enriettii*-infected guinea pigs by the injection of antilymphocytic serum (129) and suppressive parasite factors were found in the spleen of *L. donovani*-infected BALB/c mice (131). A soluble antigen of the amastigotes called "tolarogen" was thought to cause lymphocyte anergy in Ethiopian diffuse CL (130). The excess load of antigen is known to induce the proliferation of specific T-suppressor cells (132), the existence of which has been shown in experimental leishmaniasis (22,133-136).

There were certain morphological alterations in the organs of our VL cases that indicated the existence of an excess amount of antigen and immune complexes. These changes were the various manifestations (intrafollicular hyalinosis and protein droplets in the littoral macrophages of the spleen) of "tissue proteinosis" (137,138). There are only two reports in the leishmaniasis literature, as far as I know, describing the accumulation of an eosinophilic substance in the lymph follicles. Meleney found it in one of his kala-azar cases and thought it was amyloid (120). In the other report, it was described in South American kala-azar cases and regarded as secondary amyloid (139). Our polarization microscopical analysis showed, however, that it was hyaline and not amyloid. In *L. enriettii*-infected guinea pigs gamma-globulin was found in the germinal centres and the authors concluded that it was a sign of the presence of immune complexes (140). Similar intrafollicular hyalinosis has been described in other conditions (141-144) and it has been suggested that the accumulation of this material represents "a kind of central hypersensitivity reaction" to antigen freshly arrived in a follicle (145). Other investigations seem to support this theory (146,147).

The storage of protein droplets in the "littoral" macrophages of the splenic sinuses has been described in a few cases of VL (124,148), in collagen disease before the steroid era, as well as in multiple myeloma (137,138). According to Szinay's investigations, these droplets were of serum protein origin and he also cited the results of Jancsó's experiments, which suggested that the droplets were antigen-antibody complexes.

Vascular hyalinosis, which can also be regarded as a phenomenon of "tissue proteinosis" will be discussed later (page 30).

Summarizing the findings in the lymphoid organs in human VL, the following points can be made:

- (1) Selective depletion of T-dependent regions of the spleen and lymph node develops.
- (2) The reversed lymphocyte/parasitized histiocyte ratio can be observed also in kala-azar, and
- (3) intrafollicular and vascular hyalinosis, as well as the storage of protein droplets develops supporting the assumption that there is an excess amount of leishmanial antigen in kala-azar.

THE MORPHOLOGY OF MACROPHAGE-PARASITE INTERACTION IN LEISHMANIASIS

The macrophages or histiocytes are regarded today as very remarkable cells. They are not only able to phagocytose and destroy micro-organisms, other cells, and cell debris, and to take part in immunological processes by antigen-presentation, but they also secrete various substances (149-152). Mitchell in his review on host-protective immunity to parasites concluded that this immunity can be influenced by parasite-antigenicity, intramacrophage environment, and the host immune response (153). The macrophage-*Leishmania* parasite system offers a useful model for studying the second and third factors.

The ultrastructure of the parasitized macrophages has been studied in *in vitro* experiments by several workers (154-158). It has been shown that the parasites soon after internalization are tightly surrounded by a "host membrane", while later they lie in a large "parasitophorous vacuole". Ultrahistochemical studies showed that the fusion of macrophage-lysosomes with the large vacuole was not inhibited but the hydrolytic enzymes did not damage the amastigotes (155-160). The few ultrastructural reports on human CL did not show and describe large parasitophorous vacuoles, but tight "host membrane" around the parasites (71,72,160,161).

The results of our investigations showed differences on the ultrastructural level between the macrophages. There were cells which contained hypertrophied Golgi apparatus, many dilated endoplasmic reticulum channels, numerous lysosomes, and coated vesicles. The amastigotes - if there were any in these cells - were always surrounded by "host membrane"; large parasitophorous vacuoles were not observed, and degenerating parasites could be seen in these cells.

Other macrophages contained large parasitophorous vacuoles filled with viable amastigotes, granular material, and membranous bodies; the cytoplasm of these cells showed the signs of degeneration with the formation of large membranous inclusions. We believe that these differences are the expressions of the various levels of activation of the macrophages. The development of the large parasitophorous vacuole is probably the morphological sign of parasite survival in non-activated macrophages. Our immunohistological observations seem to support these assumptions, by showing strong lysozyme-positivity in non-parasitized, activated macrophages and the lack of this enzyme in the large, parasite-loaded cells. The role of macrophage activation in the killing of the parasites has also been stressed by several authors (22,101,162).

A common finding was the presence of coated vesicles in the activated macrophages. The role of these coated vesicles is not yet fully understood. They are believed to take part in enzyme transport, in membrane recycling, and in the formation of cellular interconnecting structures (163). It is known that activated macrophages have leishmania antigen on the surface of their plasma membrane (95,101-103). A constant recycling between the plasma membrane of the macrophage and the membrane of the parasitophorous vacuole has also been shown (164). One can speculate that the small coated vesicles in the activated macrophages in our studies might be one of the morphological elements of the leishmanial antigen-presenting process.

Finally, it should be pointed out that certain differences exist between our observations and those made in *in vitro* systems. These are the lack of reports in the *in vitro* experiments on membranous degenerations and on morphologic signs of enhanced metabolic activity of the macrophages (155-158). These discrepancies can be explained by the different conditions (*in vivo* vs *in vitro*) and the genetic differences between the macrophages of the different systems.

We can summarize our observations on the macrophage-parasite interaction as follows:

- (1) There is a marked difference in the ultrastructure between activated and non-activated macrophages.
- (2) This difference is manifested by the development of large parasitophorous vacuoles, the occurrence of dividing amastigotes, and the absence of necrotized parasites, the almost complete lack of signs of enhanced metabolic activity, and the ultimate lysis of the non-activated macrophages, leading to the assumptions that (a) the development of large parasitophorous vacuoles can be the

morphological manifestation of parasite survival, and that (b) the crucial factor in parasite killing is the activation of the macrophage.

THE OCCURRENCE OF "SECONDARY", LEISHMANIASIS-RELATED TISSUE ALTERATIONS IN HUMAN AND EXPERIMENTAL LEISHMANIASIS

In several tropical diseases certain organ(s) not affected primarily by the infection can be involved later, due to secondary tissue damage. Sometimes the malfunction of these organs is responsible for the dominant clinical features, and, not infrequently, can lead to death. Rupture of oesophageal varices in liver-schistosomiasis is one example of this. In other cases the abnormal response of the immune system causes serious complications mainly via circulating immune complexes. The nephrotic syndrome in leprosy (165,166), in malaria (165,167), and in schistosomiasis (168-170) is an illustration of this.

There are several reports in the literature pointing to the involvement of organs other than those of the mononuclear phagocytic system. Anaemia (27,171), nephrotic syndrome (109), and cirrhosis of the liver (172,173) can complicate the primary disease. The study of our human and experimental material revealed also the existence of secondary tissue damages such as systemic vasculitis, sclerosing glomerulonephritis, pulmonary haemorrhages, and erythrophagocytosis in human VL; local vasculitis in CL and ML; whereas local vasculitis, systemic amyloidosis, and erythrophagocytosis in the experimental animals.

Recent works have shown the presence of immune complexes in the sera of patients suffering from VL (17,174) and South American mucocutaneous or cutaneous leishmaniasis (175,176). Abdalla and associates also showed a nearly 4-fold increase of IgG and IgM in the sera of Sudanese kala-azar patients (50). Radwanski et al. demonstrated gamma-globulin in the lymph follicles, and complement in the cutaneous lesions of *L.enriettii*-infected guinea pigs and concluded that immune complexes were present in these localizations (140).

Vascular changes have been described in human CL (necrotizing vasculitis (177, 178)), in South American mucocutaneous leishmaniasis (endarteritis obliterans (179), vasculitis and fibrin thrombi (98)), in kala-azar (endothel-proliferation (139)), and in experimental leishmaniasis of monkey (120) and guinea pig (122). The occurrence of disseminated intravascular coagulation in a case of kala-azar has also been reported (180). The presence of fibrinogen around small vessels in experimental CL, a sign of enhanced permeability, has been described (140). The vascular changes we found locally in human CL and ML and in several organs in the fatal

cases of VL, as well as those we observed in the cutaneous lesions of mice and hamsters, were due to enhanced permeability (intimal oedema, fibrinoid necrosis, hyalinosis), inflammation, and the formation of fibrin thrombi. It is well known that circulating immune complexes can cause increased permeability partly by damaging directly the endothelial cells (181), and partly by a more complex mechanism via complement fixation (182). It is also known that the outcome of the formation and the localization of immune complexes depends on the absolute amounts and the relative proportions of antigen and antibody (182). Therefore we believe that the local vasculitis and thrombosis in CL and ML were the sequel of a local immunological reaction caused by an excess of antibodies, whereas the systemic vessel lesions in VL were due to circulating immune complexes associated with excess antigen, thus, providing further evidence for the role of antigen-excess in these patients (see page 27).

The rarely encountered glomerular changes in East African (109), in South American (183-185), and in our kala-azar cases can also be explained by the trapping of immune complexes in the glomeruli. This is suggested by both clinical (185) and immunofluorescence (184-196) observations.

Severe anaemia is a constant feature of human and experimental kala-azar (27,187-189). The morphological sign of RBC-destruction, erythrophagocytosis, was observed by Woodruff et al. (190) in human VL and by Agu et al. (189) in experimental *L. donovani* infection of hamsters. In kala-azar the proliferating RBC-phagocytosing histiocytes can mimic malignant histiocytosis (191). Our findings are in agreement with these observations and the enhanced production of RBCs, as shown by the hyperplastic bone marrow in all our kala-azar cases examined, is regarded as a compensation for the destruction. The anaemia is attributable to splenic sequestration and shortened life span (171,190). The binding of complement-containing immune complexes to the plasma membrane of RBCs has been shown both in human VL (188,190), and in *L. donovani* infected hamsters (189). Woodruff regards autoimmune haemolysis as the cause of anaemia in VL (192). Apart from immune complexes, there may be another mechanism causing the destruction of RBCs, namely that *Leishmania* parasites have antigen that cross-reacts with components of the RBC's plasma membrane (193).

A striking feature of the fatal kala-azar cases was the high frequency of pulmonary haemorrhages. Although thrombocytopenia, which also occurs in kala-azar (187), can cause severe haemorrhages, another mechanism can also be considered. The circulating immune complexes, the glomerular changes, the cross-reactivity between parasite's antigens and the plasma membrane of RBC's, and the lack of

haemorrhages in other organs, point to the role of an immunological mechanism as the cause of these pulmonary haemorrhages, as in Goodpasture's syndrome.

The changes of "tissue proteinosis" seen in our VL cases could not be reproduced in the experiments. Nevertheless, the deposition of another abnormal protein, amyloid, was observed in all mice and in the majority of hamsters. The polarization optical analysis proved that it was of the "secondary" type. Amyloidosis is a rarely encountered complication of kala-azar in man (5), in dog (194), and in experimental VL. It was first described in hamsters inoculated with *L. donovani* (195). This model was also used for the study of the development of amyloidosis (296-198). Amyloidosis was also found in *L. mexicana* infections (199). It is generally accepted that a connection exists between the hyperplasia of the pyroninophilic cells and amyloid SAA protein-production in the first phase of amyloidosis (200,201). The relationship between impaired cell mediated immunity and amyloidosis is not as well understood. It may be that the T-cell depletion and amyloid-formation are the expression of an immunologic tolerance (202), another possibility is the release of an amyloidogenic factor from the necrotizing lymphocytes (203), and a third explanation is that the T-cell depletion caused by antigen-overload could disturb the information exchange between T-lymphocytes and macrophages. As a consequence of this disturbance the macrophages produce AA fibrils instead of other products. The deposition of huge amounts of amyloid, can then in its turn contribute to T-cell depletion by crowding out lymphocytes from the spleen (194), thus setting up a vicious circle. Whatever the cause of amyloidosis in our experiments, the accumulation of this substance clearly indicates the abnormal function of the immune system in kala-azar.

As a non-immunological complication of human kala-azar, cirrhosis of the liver has been described in the Indian form of the disease. Goswami (172) found cirrhosis preceded by severe fatty change and periportal fibrosis in 60% of 50 biopsies. Daneshbod (173) described several Pakistani children with kala-azar and cirrhosis. He believed in a direct causal relationship between the two diseases. Other think the opposite and regard it as an incidental finding (27). Portal fibrosis has, however, been observed both in South American (204,205), and in Mediterranean (125) kala-azar. A special type of intralobular fibrosis is also known to occur in VL ("Roger's cirrhosis"(5)). We could not find any evidence of a causal relationship between VL and cirrhosis in our material, although fatty change and portal fibrosis were observed in some cases. Therefore, the possibility that some connection exists between liver damage and leishmaniasis in East Africa cannot be dismissed.

In summary:

- (1) In leishmanial infection, non-specific secondary lesions such as vasculitis, glomerular changes, pulmonary haemorrhages, and erythrophagocytosis develop in man; and vasculitis, erythrophagocytosis, and amyloidosis in experimental animals.
- (2) These alterations are probably caused by immune complexes and in case of amyloidosis by an altered function of the immune system, and
- (3) cirrhosis of the liver is not a complication of kala-azar in the Sudan.

SUMMARY

Histomorphological investigations including light-, polarization-, and electron-microscopy, immunohistochemistry, and statistical morphometric analysis were carried out on the three different forms - cutaneous, mucosal, and visceral - of Sudanese leishmaniasis, on cutaneous leishmaniasis from Saudi Arabia, and on experimental leishmaniasis of mice and hamsters infected with parasites that were isolated from Sudanese leishmaniasis patients.

The aims of the investigations were to determine the type of *Leishmania* parasite causing the various disease forms in the Sudan, to study the types of the inflammatory reaction including the immunohistology of cutaneous leishmaniasis, to analyse the histomorphology of the response of the immune system, to describe the ultra-structural features of the macrophage-parasite relationship, and to follow the secondary, non-specific, but leishmaniasis-related, alterations in the parenchymatous organs.

The study led to the following findings:

1. The numbers of subpellicular tubules did not differ significantly between the parasites from Sudanese cutaneous, mucosal, and visceral leishmaniasis. These numbers correspond well with those of *L. donovani*.
2. The inflammatory infiltrate of leishmaniasis was composed of three basic cell types: cells of the mononuclear phagocytic system, lymphocytes, and plasma cells. In addition, fairly large number of small vessels could be seen in some cases.
3. Cutaneous and mucosal leishmaniasis showed a variation in the histological picture: non-activated, parasitized macrophages were the dominating cell type in some cases; plasma cells or lymphocytes predominated in others; whereas roughly equal numbers of activated non-parasitized macrophages, lymphocytes, and plasma cells were found in the rest of the cases. Well developed, organized granulomata could be found in cases with low parasite count.
4. There was an inverse relationship between the numbers of lymphocytes and non-activated, parasitized macrophages in all forms of human and experimental leishmaniasis.

5. Immunohistochemical stainings for IgG, IgA, IgM, lysozyme, and S-100 revealed a distinct pattern in chronic non-healing cutaneous leishmaniasis in accordance with the histological picture: the predominance of IgG/IgA⁺ or lys⁺ cells, or a mixture of these cells.
6. There was a difference in the staining pattern of lysozyme between the various cells of the mononuclear phagocytic system, activated, non-parasitized macrophages showing the strongest reaction.
7. Scattered S-100⁺lys⁻ reticulum cells could be observed in the inflammatory infiltrate of chronic cutaneous leishmaniasis cases.
8. Electron microscopy revealed the presence of apoptosis in chronic cutaneous leishmaniasis.
9. In cutaneous leishmaniasis IgG⁺ and IgA⁺ cells were found scattered as single cells or in small groups within the central part of the epidermis.
10. The transformation of the histological picture from the parasitized macrophage-dominant to the lymphocyte-dominant form was observed during successful treatment in two cases of mucosal leishmaniasis; whereas mucosal leishmaniasis transformed to visceral leishmaniasis in one patient who also had tuberculosis.
11. In the fatal cases of kala-azar the histology showed the predominance of parasitized macrophages, extensive T-cell necrosis, and fibrosis of the Malpighian corpuscles. The atrophy of the Malpighian corpuscles was accompanied by depletion of thymus-dependent regions in the lymph nodes.
12. In human visceral leishmaniasis the "littoral" macrophages of the splenic sinuses contained large hyaline droplets, large amount of hyaline accumulated intrafollicularly, and there was vascular hyalinosis.
13. In experimental visceral leishmaniasis severe depletion of T-dependent regions of the spleen and lymph nodes developed and systemic amyloidosis of the secondary type was found.
14. Ultrastructural differences were observed between activated and non-activated macrophages. These were the development of large parasitophorous vacuoles, the occurrence of dividing amastigotes, the absence of necrotized parasites, and the signs of cell-lysis in the form of membranous bodies in the non-activa-

ted cells; and the signs of enhanced metabolic activity and degenerating parasites in the activated cells.

15. Intimate plasma membrane contact was seen between the activated macrophages and small lymphocytes.

Based upon these findings the following conclusions were drawn:

- a) The protozoon causing the three different forms of leishmaniasis in the Sudan is *L. donovani*, belonging probably to a subgroup of *L. donovani archibaldi*.
- b) The response of the infected host plays a decisive role in the development of the various clinical forms of leishmaniasis.
- c) The effectiveness of the host's defence is expressed in the cellular composition of the inflammatory infiltrate. Based upon the histological picture and the immunohistological patterns, four types of the inflammatory reaction can be differentiated: lymphocyte depletion, mixed cellularity, lymphocytic preponderance, and plasma cell preponderance.
- d) The activation of the macrophages is of fundamental importance in the killing of the parasites, and a well functioning macrophage-lymphocyte interaction is necessary for the activation of the macrophages.
- e) The severe T-cell depletion that can develop in leishmaniasis is probably caused by antigen-overload and circulating immune complexes.
- f) The malfunction of the immune system and an abnormal immune response, probably via the formation of immune complexes, can explain some of the complications in kala-azar, such as anaemia, nephrotic syndrome, vascular changes, pulmonary haemorrhages, and amyloidosis.

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PAPERS I - IX

MORPHOLOGICAL OBSERVATIONS ON VISCERAL LEISHMANIASIS IN THE SUDAN

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Summary. The morphological changes in 32 autopsies in subjects suffering from kala-azar are described. Splenomegaly was considerable and enlargement of the mesenteric group of lymph nodes was a characteristic feature. Histologically, L-D bodies containing cells were found in the spleen, liver, bone marrow, lymph nodes and occasionally in the intestines, lungs and kidneys. The splenic and lymphoid follicles usually showed atrophy and accumulation of intrafollicular hyaline material. The mesangial space and the basement membrane of the glomerular tufts were found to be thickened. In the small muscular arteries of the different organs plasma and hyaline material was deposited in the subendothelial space. These alterations (follicular atrophy, glomerular and vascular changes as well as hyaline accumulation) may be manifestations of an immunological process. A remarkable feature was the hypercellularity of the bone marrow in all cases. No direct causal connection between liver cirrhosis and the Sudanese (African) type of visceral leishmaniasis could be established.

INTRODUCTION

Visceral leishmaniasis or kala-azar is endemic in certain areas of the Sudan, mainly along the Blue Nile, and epidemic flares occur from time to time (Archibald and Mansour, 1937; Satti, 1958). Although more than 30 communications dealing with the different aspects of kala-azar in the Sudan have been published since the disease was first described in this country by Neave in 1904, none of them dealt in any detail with pathological changes. It was, therefore, thought worthwhile to report on the pathological changes in visceral leishmaniasis in autopsy material.

MATERIAL AND METHODS

The material consists of a study of 32 autopsies in the Pathological Department of the Faculty of Medicine, University of Khartoum, between the years 1959 – 1971. This department serves Khartoum Civil Hospital, which is the largest and principal reference hospital in the Sudan, receiving patients from all over the country. The clinical and morbid anatomical data were obtained from the autopsy records. Histological sections taken from the different organs were reviewed. Paraffin sections were stained with haematoxylin and eosin, PAS-galloycyanin or PAS-alcian blue, Azan PTAH, Giemsa, Leishman staining, Congo red, methyl violet and silver impregnation.

RESULTS

Of the 32 patients, 31 were males. Their average age was 25.5 years, the youngest being 15 and the oldest 45. Twenty of the patients were from Southern Sudan, the remaining 12 from North. Of four no history was known.

Clinically 13 were recognized as cases of kala-azar during life and given the appropriate treatment; the remainder died undiagnosed though most of them had had one or more clinical features of visceral leishmaniasis (*Table*). The duration of the illness varied from 10 days to five months. The diagnosis was confirmed by demonstration of L-D bodies, in the bone marrow or lymph node or spleen.

TABLE
SIGNS IN 28 CASES OF KALA AZAR

Signs	Fever	Hepatomegaly	Splenomegaly	Lymphadenopathy	Haemorrh.: diathesis	Anaemia	Leukopenia
No.	26	22	27	9	10	29	15

Gross and histological changes.

Liver. Some degree of liver enlargement was a usual finding. The average weight was 1715 g. With the exception of one case who had an associated cirrhosis, the liver surface was smooth. The organ was firm in consistency and occasionally showed passive venous congestion. Histologically, L-D bodies were seen either in the Kupffer cells (*Fig. 1*), or in the histiocytes in the portal tracts, or in both in 21 out of 27 cases (in five cases out of 32 no sections were available). In the remaining six cases no L-D bodies could be detected in histological preparations: five out of these six cases had been treated during life. The Kupffer cells were enlarged, sometimes filling the sinuses completely (*Fig. 1*). Some of them were vacuolated or degenerated. In some of those cells where no L-D bodies were seen, the small vacuoles contained eosinophilic granules. In the sinuses lymphocytosis was noted (*Fig. 1*). The parenchymal cells did not contain any L-D bodies; however, they showed some degree of atrophy in 13 cases while some fatty change — mainly in the periphery of the lobule — was seen in nine cases. Focal necrosis occurred in five cases in association with chronic venous congestion. All cases showed some degree of periportal chronic inflammatory infiltration consisting mainly of lymphocytes and plasma cells (*Fig. 1*). Fibrous thickening of the portal tract was noted in six cases only and was not severe in any of them; it was observed that in four of these six cases the number of Kupffer cells containing parasites was high. Cirrhosis of portal type was found only in one case.

Spleen. In all cases the spleen was considerable enlarged; the average weight was 880 g. Its surface was smooth and normal-looking with the exception of one case that showed perisplenitis. On the cut surface there was the striking feature of atrophy or even absence of the Malpighian corpuscles. The splenic pulp was red, increased in amount and friable. In some cases infarctions and haemorrhages were found. In 20 cases L-D bodies were detected in histiocytes; in six cases sections did not reveal L-D bodies; five of these cases had been treated during life. In the remaining six cases histological preparations were not available, but smears were done at autopsy and five of these cases were positive. Parasitized histiocytes were found mainly in the pulp although some were seen in the Malpighian corpuscles as well. A remarkable histological feature was the atrophy of the Malpighian corpuscles, seen in 18 cases. There was accumulation of extracellular material in the splenic follicles in 14 cases; this material was homogeneous, eosinophilic, PAS positive, stained blue with Azan and yellow with PTAH; it did not stain with Congo red and was not metachromatic (*Fig. 2*). Chronic inflammatory cells, mainly plasma cells, were observed in the pulp.

Lymph nodes. At autopsy lymphadenopathy was noted in 28 cases. The mesenteric lymph nodes were enlarged in 23 cases.

Microscopically the architecture of the lymph node was preserved, L-D bodies were seen in 18 cases; nine cases were negative while in the remaining five no histological preparations were available. The affected lymph nodes showed sinus cell hyperplasia with increase and activation of sinus endothelial cells and scattered plasma cells in the medulla. In 15 cases the lymph follicles were atrophic. In six cases there was accumulation of extracellular material in the follicles with similar characteristics to that seen in the spleen. In one case there was marked fibrosis with evidence of lymph stases (dilated lymph vessels and interstitial oedema); in this case, however, an accompanying bilharzial infection of the liver was noted.

Bone marrow. The bone marrow was examined in 15 cases, five of them at autopsy. L-D bodies were detected in histiocytes in all of them. On histological examination hypercellularity was observed in most cases.

Intestines In two cases the mucosa of the small and large intestine showed hyperaemia, thickening, false membranes and ulcers. Histologically in these cases the villi were oedematous and the lamina propria was infiltrated by chronic inflammatory cells together with parasitized histiocytes. The superficial part of the mucosa was necrotized and fibrin-rich exudate covered the surface.

Lungs. In seven cases multiple haemorrhages were seen. In four cases the lungs showed evidence of interstitial pneumonitis with thickening of the alveolar walls caused by mononuclear cell infiltration and capillary dilation. L-D bodies in histiocytes were detected in two of these four cases.

Kidneys. Macroscopically the kidneys looked normal or slightly congested, except in two cases, one of which showed the features of acute pyelonephritis and the other of chronic. Although L-D bodies in histiocytes were detected in only one case, significant changes in the glomeruli were noted in 18 cases. These lesions varied in severity. They were usually diffuse. The epithelial cells of the glomerular tufts were swollen, their nuclei being rather pyknotic and the cytoplasm more or less homogeneous and eosinophilic. In some glomeruli there was actual increase in the number of the epithelial cells due to their proliferation. The mesangial space was widened by the accumulation of eosinophilic material. The capillary basement membrane was occasionally thickened as shown by reticulin staining (Fig. 3, 4). There was some eosinophilic material in the Bowman's capsule and casts in the tubules. In one case there was pyelonephritis of acute type, while in another case there was chronic pyelonephritis.

Heart. In two cases the myocardial fibres in the subendocardial region showed swelling and vacuolation associated with the arterial changes described below.

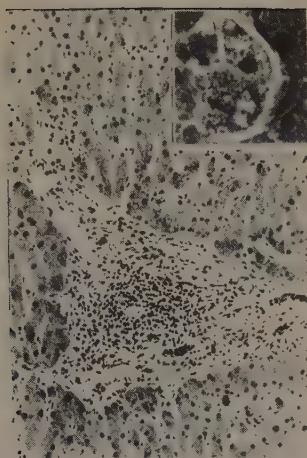
Vascular changes. These were observed in 12 cases, affecting mainly the small muscular arteries in the adrenal capsule, liver, kidney, heart and intestine. Only four out of the 12 cases were treated during life. The vascular alterations consisted of vacuolation and swelling of the endothelial cells and focal thickening of the intima resulting from accumulation of plasma and hyaline-like material in the subendothelial space (Fig. 5, 6); this material was PAS positive and stained blue with Azan and yellow with PTAH. There were no associated hypertensive or arteriosclerotic changes in these cases.

DISCUSSION

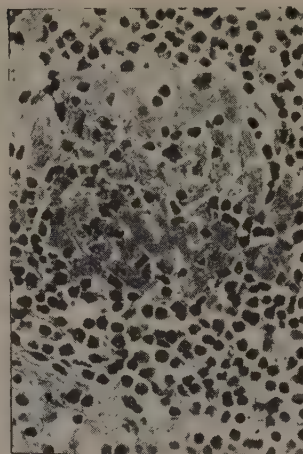
The Sudanese form of visceral leishmaniasis is the African type, which differs from the Mediterranean and Indian type in certain epidemiological and clinical respects (Edington and Gilles, 1969). Thus, whereas the Mediterranean type occurs mainly in children up to 13 years of age (Yasarol, 1965) and the Indian type has a peak incidence at 10–20 years (Edington and Gilles, 1969), the African type is commoner in adults than in children (Manson-Bahr and Heisch, 1956; Satti, 1958; Takle *et al.*, 1970). In our series the average age was 25.5 years with 56.6% of the cases between 20 and 29 years.

Our results show that the commonest clinical features were fever, anaemia, splenic enlargement and hepatomegaly. Leucopenia and haemorrhages – including epistaxis, haematemesis, melaena, bleeding from gums and pulmonary haemorrhages – were also fairly frequent. Lymphadenopathy was noted clinically in nine cases only but was found to be more widespread in 23 cases at autopsy. It was interesting that in one case L-D bodies were only found in the lymph nodes. Taking this in conjunction with what Sati (1958) has referred to as 'localized leishmaniasis in lymph nodes', it becomes obvious that lymph gland biopsy when marrow and splenic punctures are negative may still clinch the diagnosis in clinically suspicious cases. It was interesting to notice that out of the six cases in which the microscopical examination did not show L-D bodies containing cells five had been treated with antimony compounds during life.

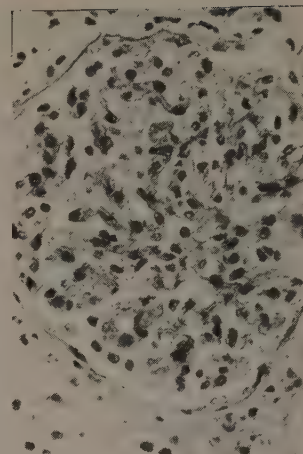
The causative agent of visceral leishmaniasis is the protozoon *Leishmania donovani*



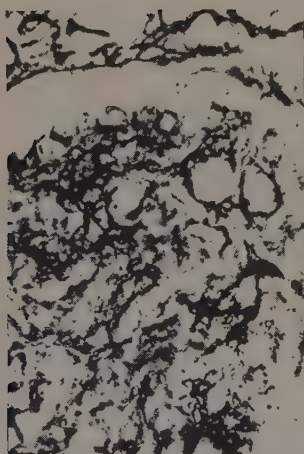
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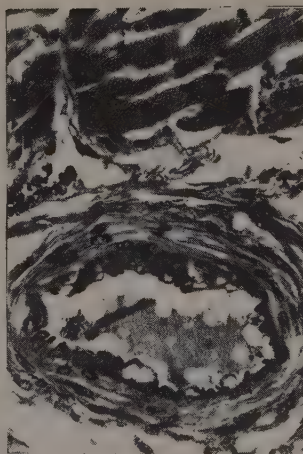
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Fig. 1. Chronic periportal inflammatory infiltration, enlarged Kupffer cells and sinus lymphocytosis. Insert shows L-D bodies in Kupffer cells. H & E; x 60 and 240.

Fig. 2. Eosinophilie, band-like material accumulated in the Malpighian corpuscle. H & E; x 240.

Fig. 3. Mesangial thickening and pyknotic nuclei of the epithelial cells are seen. H & E; x 192.

Fig. 4. Thickened basement membrane in a glomerulus. Reticulin; x 240.

Fig. 5. Eosinophilic material is deposited in a small coronary artery. H & E; x 180.

Fig. 6 (upper). Homogeneous hyaline material seen in the subendothelial space of a small artery from the periadrenal tissue. H & E; x 180.

Fig. 6 (lower). The accumulated substance is PAS positive in a hepatic artery. PAS-gallocyanin; x 156.

which affects the reticulo-endothelial system. In our autopsy material splenic enlargement was the rule, hepatomegaly was not so remarkable and mainly the mesenteric group of lymph nodes was enlarged.

Histologically, apart from the presence of L-D bodies in histiocytes and Kupffer cells, two other lesions were remarkable: (a) atrophy of the Malpighian corpuscles in the spleen and of the lymphoid follicles in the lymph nodes and (b) the extracellular deposition of hyaline material in these structures. Edington and Gilles (1969) had remarked that these follicular changes were unusual but we found them in 18 spleens (69.2%) and 15 lymph nodes (55.5%). Meleney in 1925 had reported such atrophic lesions in the lymphoid follicles in kala-azar and one of his cases showed accumulation of eosinophilic material 'suggestive of amyloid' in the Malpighian corpuscles. He had not explained these phenomena, however. Andrade and Andrade (1966) in a study of 13 autopsies also found deposition of hyaline substance in connective tissue 'distributed as in secondary amyloidosis'. Cooper *et al.* (1969) concluded that the hyaline material was brought about by 'increased vascular permeability and represented a kind of central hypersensitivity reaction, elicited by the arrival of fresh antigen in a follicle already producing reciprocal antibody'.

Although albuminuria is known to be a common feature of kala-azar, its pathogenesis has not been adequately worked out. In African kala-azar, Manson-Bahr and Heisch (1956) described associated renal changes with membranous glomerulonephritis. Andrade and Iabuki (1972) in a study of 18 autopsies, described fibrillar mesangial thickening with swelling and proliferation of glomerular epithelial cells. They postulated that the cause of the glomerular changes was the trapping of globulin in the mesangial matrix. In our series, the glomeruli in 16 out of 27 cases showed not only epithelial changes and mesangial alterations similar to those described by Andrade and Iabuki (1972) but also occasional thickening of the basement membrane.

In the blood vessels proliferation of the endothelial cells was found both in mucosal and visceral leishmaniasis (Meleney, 1925; Klotz and Lindenberg, 1923; Perry, 1922) resulting in partial vascular occlusion. Vascular alterations affecting the small muscular arteries in our series consisted of vacuolation and swelling of the endothelial cells and subendothelial accumulation of plasma and hyaline-like material.

We believe that these changes — the atrophy of the follicles and the hyaline deposition, the renal and vascular alterations — are manifestations of an immunological process. Recently the role of immunity in the development and course of leishmaniasis has been stressed by Bryceson *et al.* (1970) and Heyneman (1971). The role of immunity in kala-azar is also reflected in the cellular reaction consisting of a proliferation of histiocytes and secondary infiltration by lymphocytes and plasma cells which is widespread throughout the viscera, particularly in the spleen, liver, bone marrow and lymph nodes (Adler, 1964). Circulating antibodies have also been demonstrated by complement fixation tests (Sen Gupta and Adhikari, 1952; Heyneman, 1971).

Formerly, it was generally thought that there was decreased cellularity of the bone marrow in kala-azar (Edington and Gilles, 1969). In our series, however, most of the bone marrows examined showed hypercellularity. This aspect is now the subject of further investigation.

The association of liver cirrhosis and leishmaniasis has been the subject of controversy. Goswami (1970) in a study of hepatic needle biopsies in 50 kala-azar patients in India, found that 60% showed evidence of cirrhosis, this being preceded by fatty change and periportal fibrosis. He concluded that the liver changes and the development of cirrhosis in kala-azar was just a matter of time. Daneshod (1972) also found liver cirrhosis in Pakistani children five months after the infection; he also stressed the direct causal

relationship between kala-azar and cirrhosis. On the other hand, Meleney (1925) did not find any evidence of cirrhosis in his cases and according to Edington and Gilles (1969), 'cirrhosis, if present, must be considered an incidental finding'. Chadli and Philippe (1961) from Tunisia found only periportal infiltration but no fibrosis or cirrhosis. Da Silva and de Paola (1961), in a study of liver needle biopsies from 30 kala-azar patients, also found no evidence of cirrhosis, although they had earlier reported that fibrosis was a constant feature (Da Silva and de Paola, 1958).

As regards our own material, cirrhosis was found in only one case and periportal fibrosis – which was mild – in only six cases. The reticular network of the liver in these latter cases was not disturbed. It seems to us that *Leishmania donovani* affects only the Kupffer cells and included an inflammatory reaction which, however, is not so severe as to damage the hepatic parenchymal cells or distort the hepatic architecture.

Involvement of the other organs was not frequent in our cases. Interstitial pneumonitis was noted in four cases; this was also reported by Andrade and Andrade (1966) who considered it to be a non-specific lesion. The intestine was affected in two of our cases – ulcerative, diphtheroid inflammation similar to that reported by Perry (1922), Meleney (1925), Sati (1962), Prevot *et al.* (1968) and Takle *et al.* (1970).

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Morphology of the spleen and lymph nodes in fatal visceral leishmaniasis

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Summary. Histological appearances of the spleen and lymph nodes were analysed in twenty fatal cases of human visceral leishmaniasis from the Sudan. Marked atrophy of the splenic white pulp was associated with necrosis and fibrosis of thymus-dependent areas, accumulation of parasite-containing histiocytes and plasma cell hyperplasia. Depletion of small lymphocytes in the paracortical areas of the lymph nodes was accompanied by proliferation of plasma cells and histiocytes in the paracortex. Depletion of small lymphocytes in thymus-dependent regions of lymph nodes and spleen is viewed as arising from immune suppression associated with antigen overloading or other factors, which may impair those aspects of lymphocyte-macrophage cooperation that are presumably necessary to kill the invading parasites.

INTRODUCTION

The study of immunological responses in different forms of leishmaniasis is of past and present interest. Thus in endemic areas of East Africa it has long been customary to inoculate children with materials taken from leishmanial ulcers to prevent the development of scars due to natural infection (Manson, 1914). In Kenya, immunity is said to last for at least

10 years following recovery from kala-azar (Manson-Bahr, 1959, 1961); and cellular immunity is viewed as a main defence mechanism against the leishmanial parasite (Adler, 1963; Bryceson, Bray, Wolstencroft & Dumonde, 1970; Stauber, 1970; Heyneman, 1971; Bray, 1972; Woo, 1974) as in certain other tropical, helminthic and fungal diseases (Turk, 1969, 1970; Turk & Waters, 1968, 1971; Keller & Keist, 1972; Krupp, 1974; Purtilo, Meyers & Connor, 1974; Carrada-Bravo, 1975).

In leishmaniasis, immunological and morphological studies have generally been undertaken in animals and in human cutaneous forms of the infection (Bryceson, 1969, 1970; Bryceson *et al.*, 1970; Heyneman, 1971). To our best knowledge, the morphological interpretation of changes in the spleen and lymph nodes in human kala-azar (Meleney, 1925) has not yet been referred to modern immunological concepts. This paper describes the examination of twenty fatal cases of visceral leishmaniasis and reports a depletion of small lymphocytes in thymus-dependent areas both in spleen and lymph nodes accompanied by an abundance of parasite-containing histiocytes and hyperplasia of plasma cells.

MATERIALS AND METHODS

Spleens and lymph nodes were obtained from twenty post-mortem cases of visceral leishmaniasis examined at the Pathology Department, University of Khar-

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toum. Clinical and other morbid anatomical data were obtained from the records. Paraffin-embedded sections of the spleen and lymph nodes were stained with haematoxylin and eosin, Giemsa, Leishman, PAS and reticulin stains for light microscopical investigation; and with Congo red (Romhanyi, 1971) for polarization microscopy.

Morphometric analysis of splenic sections was carried out on haematoxylin-eosin stained sections from two blocks of each case. The area of the whole section was first determined by projection of the slide on to the screen of a Reichert-Visopan microscope equipped with millimeter paper. After having drawn the borders of each section the number of square millimeters was counted and finally the area of the section and the white pulp area were expressed in square microns. The area of the Malpighian corpuscle was evaluated by subtracting the projected area of the central artery from that of the total white pulp area. For comparative purposes a ratio of white pulp over total spleen area (WP/S) was calculated and referred to a unit spleen area of $1 \times 10^6 \mu\text{m}^2$. A control group of sections from lymph node and spleen was obtained from fifteen subjects who died without a history of disease (e.g. car accident; murder) and were selected from the same age group as those with leishmaniasis. The total number of individual white pulp areas measured were 962 and 574 in the kala-azar and in the control groups, respectively.

RESULTS

In the leishmaniasis group all twenty patients were males; the average age was 25 years (range: 16–45 years); leucopenia was regularly observed (average white blood cell count: $4300/\text{mm}^3$) but relative lymphocytosis was absent in sixteen cases. In all patients the spleen was considerably enlarged (average weight 860 g). The immediate cause of death was sepsis (including two cases with secondary fungal lung infection), bronchopneumonia and miscellaneous conditions such as uremia, haemorrhagic shock and amoebiasis in 11, 4, 2, 2 and 1 patients, respectively. In five of the patients leishmaniasis had been diagnosed prior to death and had been treated for periods of 7–16 days.

Morphometric analysis of the spleen in kala-azar revealed significant reduction ($p=0.02$) in the size of the white pulp areas ($10.72 \pm 6.52 \times 10^3 \mu\text{m}^2$) as

compared to that of the control group ($29.38 \pm 5.7 \times 10^3 \mu\text{m}^2$). In the control spleens histology showed densely packed lymphocytes around the central arteries of the Malpighian corpuscles, whereas in none of the twenty cases of kala-azar studied were any germinal centres seen and the white pulp itself was loosened and disorganized (Fig. 1). In six out of the twenty cases central necrosis was found destroying the normal architecture of the white pulp (Fig. 2). The number of lymphocytes was invariably very low in all white pulps examined; and occasionally, the lymphocytes were virtually missing, being replaced by plasma cells and parasite-containing histiocytes (Fig. 3). Fibrosis of the white pulp was sometimes observed (Fig 4). A striking feature was the accumulation of an eosinophilic, PAS-positive material in the extracellular space of the follicles. This material did not show birefringence under polarized light. In the red pulp, large numbers of plasma cells and parasitized histiocytes were seen as well as proliferation of the sinus endothelial cells. In the cytoplasm of several histiocytes, fragments of red blood corpuscles and haemosiderin pigment were found.

In the lymph nodes of kala-azar patients there was a PAS-positive substance deposited in the germinal centres of the primary follicles similar to that described in the spleen (Fig. 5). The paracortical areas appeared pale at low power view due to the proliferation of parasite-containing histiocytes with occasional plasma cells and large lymphoid cells replacing the missing small lymphocytes (Fig. 6). Epithelioid cells were absent. The medullary cords were packed with plasma cells and histiocytes. In eight out of twenty cases the histiocytes contained fragments of erythrocytes.

DISCUSSION

During the last decade considerable progress has been made in interpreting the histopathology of various tropical diseases accompanied by abnormal immune status. Thus Turk and his colleagues showed that lepromatous leprosy was regularly accompanied by lymphocyte depletion in the paracortex of lymph nodes, whereas no such paracortical depletion was seen in the tuberculoid form of the disease (Turk & Waters, 1968; Turk, 1969). Similar results were obtained in experimental leprosy (Hangen, Skjorten & Closs, 1975; Pfak, Gaugas, Rees &

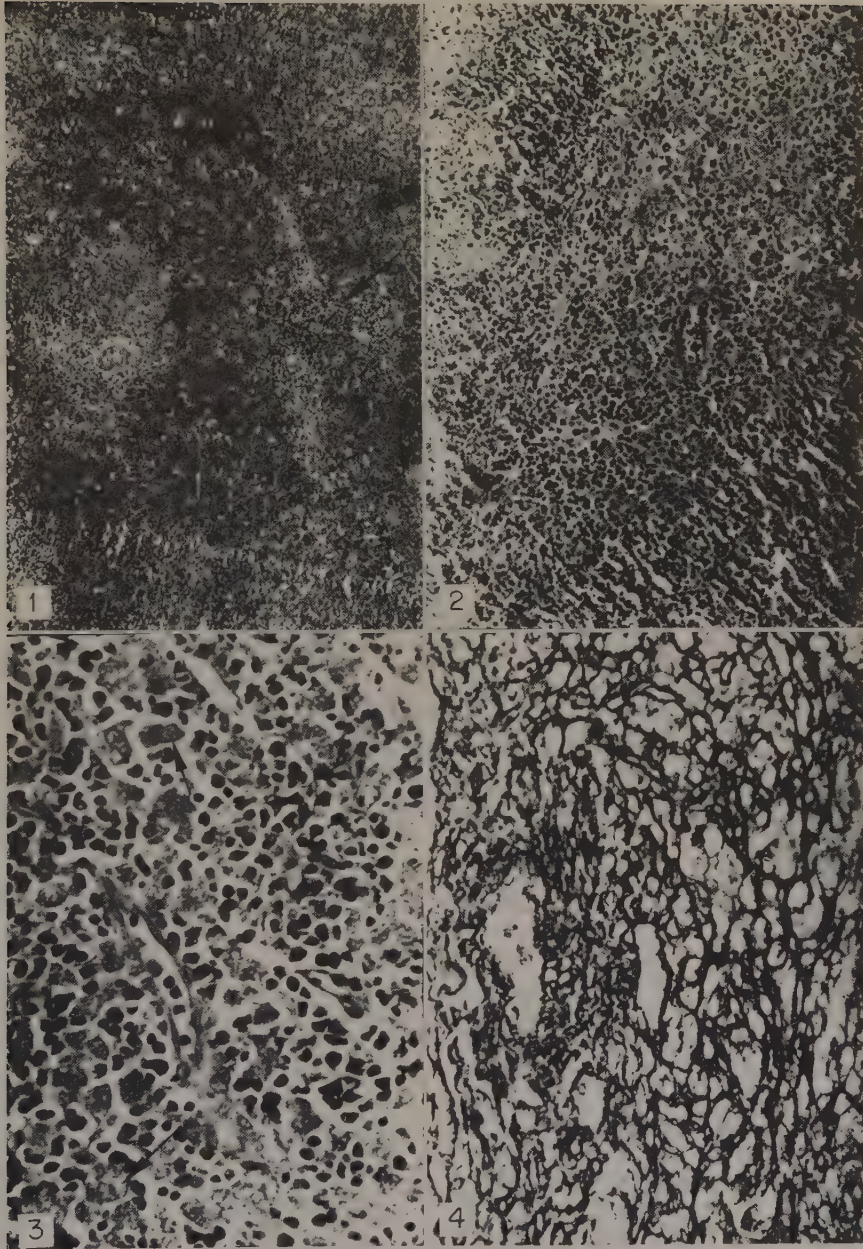


Figure 1. The Malpighian corpuscles are small (arrow) and their architecture is disorganized (double arrow) (H & E, $\times 60$).
Figure 2. The number of lymphocytes diminished and large areas show shadows and remnants of necrotic cells (H & E, $\times 100$).
Figure 3. Detail of a Malpighian corpuscle. The lymphocytes are replaced by plasma cells and parasitized histiocytes (arrow) (H & E, $\times 220$).
Figure 4. Silver impregnation reveals marked fibrosis in the Malpighian corpuscle (Silver impregnation, $\times 180$).

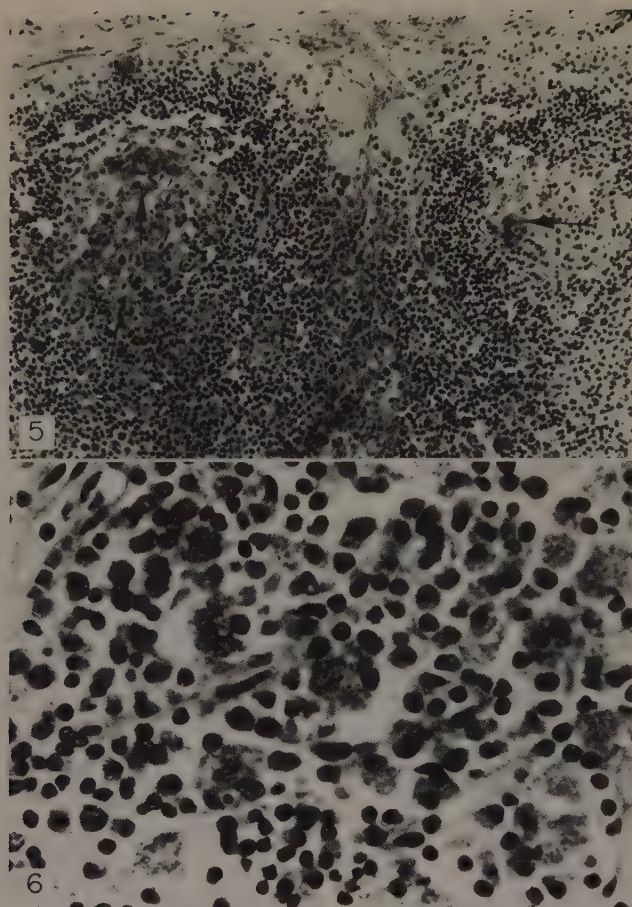


Figure 5. Detail of a lymph node. In the primary follicles eosinophilic materials are deposited (arrow) (H & E, $\times 100$).

Figure 6. In the paracortical area of a lymph node the small lymphocytes are partly replaced by plasma cells and parasitized histiocytes (H & E, $\times 300$).

Allison, 1970). In experimental immunology morphometric methods have been used to relate the white pulp areas of the spleen with immunological responsiveness (Graber & Corvazier, 1960; Feher & Nagy, 1972; Hard, 1974) but such morphometric methods have not previously been applied to the study of human spleen in tropical diseases which are known to be accompanied by immunological abnormality.

In the present study, morphometric analysis of spleen white pulp areas, together with the lymph node morphology were suggestive of a profound disturbance in cell-mediated immunity in fatal kala-

azar. Our investigations revealed marked reduction in size of the white pulps due to a loss of small lymphocytes, with necrosis and fibrosis of the central areas of the follicles. This process was accompanied by the proliferation of parasite-containing histiocytes and plasma cells both in the white and red pulps of the spleen and in the paracortical areas of the lymph nodes. These findings extend the observations of Meleney (1925) who described splenic follicular atrophy in kala-azar accompanied by the accumulation of an intra-follicular eosinophilic substance, which we and others have also observed (Andrade & Andrade,

1966; Veress, Malik, Satir & El Hassan, 1974). In experimental kala-azar, Stauber (1958, 1970) described atrophy of the splenic follicles due to a loss of small lymphocytes and to an increased number of macrophages and plasma cells; and human kala-azar is viewed as associated with suppression of specific cell-mediated immunity (Bryceson *et al.*, 1970; Turk, 1970).

Histological depletion of thymus-dependent regions in kala-azar might be caused by two mechanisms. The first one might be the appearance of an antilymphocytic antibody causing selective necrosis observed in the splenic follicles. This suggestion arises from the findings of Bryceson & Turk (1971) who produced paracortical depletion in the regional lymph nodes of guinea pigs by the injection of anti-lymphocytic serum into animals bearing experimental infections with *Leishmania enriettii*. The possible pathogenetic role of antilymphocytic antibodies has been suggested in syphilis (Levene, Turk, Wright & Grimble, 1969; Turk, 1970) and in systemic lupus erythematosus (Horwitz & Cousar, 1975). A second mechanism might be the overloading of the body by leishmanial antigen as described for lepromatous leprosy where there is a deficiency of specific cell-mediated immunity (Turk, 1969). In diffuse cutaneous leishmaniasis, Bryceson (1970) suggested that cellular anergy was caused by the over-production of a soluble leishmanial antigen ('tolerogen') which would specifically interfere with the cellular immune response. The morphological manifestation indicating such 'antigen-overloading' in kala-azar might be the accumulation of the intrafollicular hyaline substance. In experimental immunization, van Rooijen (1973, 1975) showed that labelled antigen was trapped by the splenic follicles; and Cooper, Haq & Baquell (1969) concluded that intrafollicular hyaline deposits were due to antigen-antibody complexes precipitated by excess antigen. In kala-azar a further immunological abnormality is suggested by erythrophagocytosis observed in the spleen and lymph nodes; this would indicate pathological destruction of erythrocytes and would support Woodruff's (1972) theory of the autoimmune nature of anaemia in kala-azar.

Our histological observations support the view that cooperation between (T)-lymphocytes and macrophages is important for effective killing of the invading *Leishmania*, as in other related diseases (Bryceson, 1969; Turk & Waters, 1971; Farah, Samra & Nuwayri-Salti, 1975). The abundance of

parasite-containing histiocytes in these fatal cases indicated the ability of these cells to phagocytose but not to kill *Leishmania*; and a similar phenomenon has been described in lepromatous leprosy (Turk & Waters, 1971; Hangen *et al.*, 1975). This finding might be explained either by lymphocyte depletion or by interference by excess antibody with macrophage membrane function (see Watson, Slijvic & Brown, 1975). Our observations raise the desirability of carefully investigating the immunological status of patients with kala-azar, before, during and after treatment.

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IMMUNOHISTOLOGICAL INVESTIGATIONS IN CHRONIC CUTANEOUS
LEISHMANIASIS IN SAUDI ARABIA

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SUMMARY

The distribution and numbers of IgG-, IgA-, IgM-, and lysozyme-positive cells were investigated by the immunoperoxidase method in paraffin-sections of 13 cases of chronic cutaneous leishmaniasis of low parasite load from Saudi Arabia. The majority of the peroxidase-positive plasma cells contained IgG, whereas the numbers of IgA+ and IgM+ plasma cells were not so numerous. Small groups of squamous epithelial cells showed immunoreactivity for IgG and IgA. Similar positive staining was observed extracellularly in the oedematous upper dermis, in the endothelial cells, and in the perivascular space. The activated macrophages showed strong and diffuse peroxidase staining for lysozyme, whereas epithelioid cells and multinucleated giant cells were negative or had finely granular and considerably weaker staining. It is suggested that humoral immunity also participates in the elimination of the parasites and an immunologically induced necrosis might be responsible for the ulceration of the skin in cutaneous leishmaniasis. It is also assumed that the lysozyme immunoreactivity can be a marker of the activation state of the macrophages.

INTRODUCTION

Leishmaniasis is a protozoal disease and considered second in importance to malaria (1). The protozoon *Leishmania* is an obligate intracellular parasite living in the cells of the mononuclear phagocytic system. The disease occurs in three main forms: visceral, muco-cutaneous, and cutaneous, each caused by different species or subspecies of the parasite (2). Because cutaneous leishmaniasis is widely distributed in the tropical and subtropical regions of the world and because it is the easiest to biopsy, it has been the form most studied by the light microscope and a few immunohistochemical investigations have also been carried out (3-7). Apart from these publications two papers dealt with the identification of *Leishmania* parasites by the immunoperoxidase method (8,9); and one paper described gamma-globulin, fibrinogen, and complement in experimental leishmaniasis (10). The aim of our study was to investigate the distributions and numbers of IgG-, IgA-, IgM-, and lysozyme-positive cells in 13 cases of chronic cutaneous leishmaniasis of low parasite load.

MATERIALS AND METHODS

The patients were seen in the Dermatology Clinic, King Fahd Hospital, Hoffuf, in the Eastern Province of the Kingdom. They were aged between 3 years and 45 years. There were 11 males and 2 females. The duration of the disease was more than one year in each case. The patients had no treatment and none of the lesions showed any signs of healing. One half of a 4 mm punch biopsy from the edge of the lesion was fixed in a formaldehyde - mercuric chloride - glacial acetic acid fixative and embedded into paraffin. The other half of the material was processed for electron microscopy. Paraffin sections were stained with haematoxylin and eosin, and Giemsa. The parasite index was determined according to Ridley and Ridley's method (11).

Immunoperoxidase staining techniques were carried out on serial sections to localize IgG, IgA, IgM, and lysozyme with "Histoset" Immunoperoxidase Kit (Ortho Diagnostic Systems Inc., Raritan, N.J., U.S.A.). In negative parallel control sections from each case in each bath non-immune serum replaced the specific anti-globulin or anti-lysozyme serum. Normal human skin from 5 persons was also used as a control for both positive and negative immunoperoxidase reactions. Positive control sections of tonsil were included in each bath of staining. Five fields of each immunoperoxidase stained sections were chosen at random from the diseased areas at x500 (objective: Zeiss Plan 40/0.65; eye piece: Zeiss Kpl.W x 12.5) and the number of positive cells was counted.

RESULTS

Light microscopy of H & E, and Giemsa stained sections showed hyperkeratosis and acanthosis of the epidermis with occasional basal liquefaction. The parasite index was 1+ in 11 cases and 2+ in two cases. The inflammatory infiltrate consisted predominantly of histiocytes, plasma cells, and lymphocytes in 3, 5, and 2 cases, respectively. In the remaining three cases there was a roughly equal proportion of these cells. Necrosis of disseminated, single histiocytes occurred in 10 cases, whereas large focal necrosis was found in three cases.

The distribution of the various immunoglobulin classes. The vast majority of the peroxidase-positive plasma cells and lymphoplasmacytic cells contained IgG (mean number: 225 cells/5 HPF). The numbers of IgA⁺ and IgM⁺ cells were not so numerous (mean number: 45 IgA⁺ and 30 IgM⁺ cells/5HPF). In two cases no IgM⁺ cells

were found. Several strongly IgG- or IgA-immunoreactive squamous epithelial cells were found in small groups within the prickle-cell and granular layers in all cases (Fig. 1). If there was oedema in the upper dermis, a diffuse positive staining for all immunoglobulin classes could also be seen in the interstitium. The endothelial cells of some of the small vessels and the perivascular space showed occasionally a moderately strong staining for IgG.

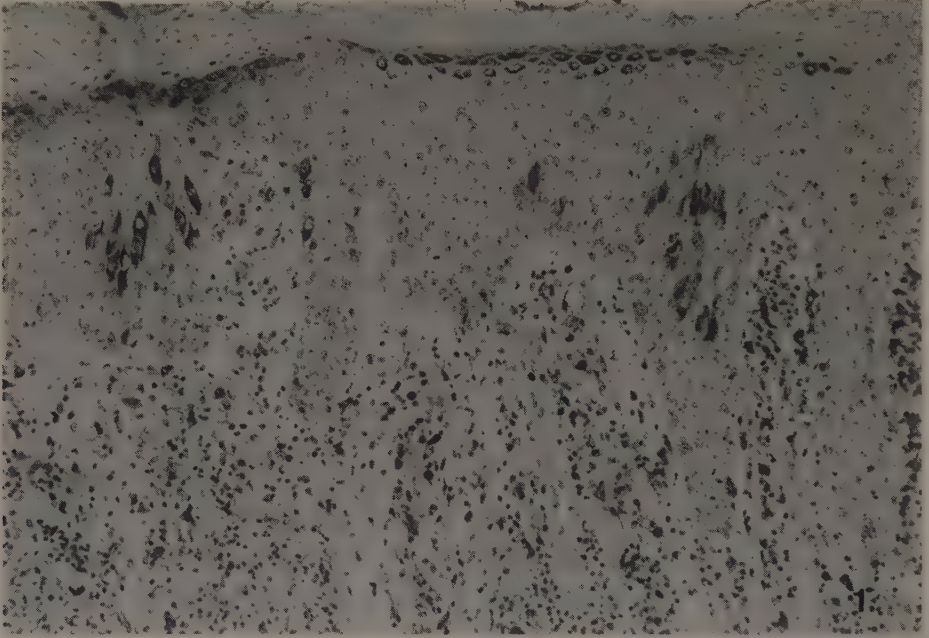


Fig. 1. Case 7/83. Groups of squamous epithelial cells and lymphoplasmacytic cells show positive staining after peroxidase reaction for IgA. (x40).

Negative control sections from these cases and the sections from normal human skin showed no immunoglobulin-positive cells in the epidermis, in the dermis, or in the vessels.

The distribution of lysozyme. The strength of the lysozyme staining and the numbers of positive cells varied. In the three cases of histiocyte-predominance there were numerous lys⁺ macrophages (mean numbers: 192/5 HPF); whereas in the other cases the mean numbers of these cells were less, 51 cells/5 HPF. It was interesting to note that not all of the macrophages and epithelioid cells showed lysozyme-immunoreactivity. The small mononuclear cells and the larger activated macrophages showed a strong and diffuse peroxidase reaction in the cytoplasm. Parasitized macrophages were always negative. Some of the epithelioid cells showed a weak and granular reactivity, but many of these cells were negative (Fig. 2). Some of the multinucleated giant cells showed granular, weak staining (Fig. 3), while others

did not show any positivity. The epidermis and the dermis were always lys^- . No staining was found in the negative control sections.

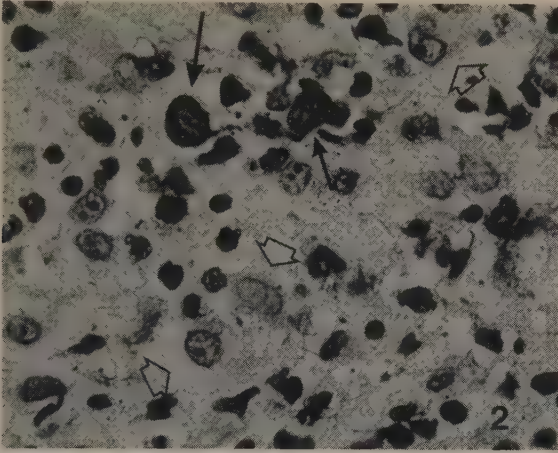


Fig. 2. Case 3/83. Some of the macrophages show very strong, diffuse staining for lysozyme (dark arrows), whereas the epithelioid cells are negative (open arrows). (x160).

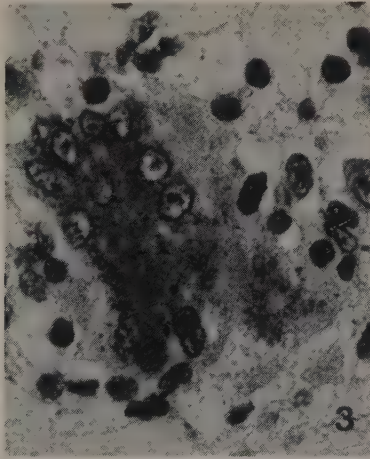


Fig. 3. Case 4/84. Moderately strong, granular staining for lysozyme in the cytoplasm of the giant cell. (x160).

DISCUSSION

An immunohistological analysis of the occurrence and distribution of IgG, IgA, and IgM in 13 non-healing, chronic cases of cutaneous leishmaniasis with low parasite load revealed that the majority of the peroxidase-positive plasma cells contained IgG, whereas the numbers of IgA^+ and IgM^+ plasma cells were lower. In two cases, no IgM^+ plasma cells were found. The low IgM-immunoreactivity was not expected

because earlier investigations had shown a marked increase of IgM in the sera of Sudanese cutaneous leishmaniasis patients (12). The predominance of peroxidase-positive plasma cells in several cases and their relatively large numbers in the remaining cases suggest that the humoral immunity has a role in the elimination of the parasites. This is in accordance with the findings of Ridley and Ridley (6) who have shown a rise of immunoglobulins, particularly IgG, in the acute phase of cutaneous leishmaniasis parallel with the fall of the parasite load to zero. Sharp elevation of leishmania-specific antibodies of several IgG subclasses (IgG1, IgG2a, IgG3) and IgM were detected in experimental leishmaniasis (13) and it was suggested that antibodies influence the binding and phagocytosis of parasites by macrophages (14).

Several squamous epithelial cells, the oedematous upper dermis, the endothelial cells, and the perivascular space also showed positive peroxidase staining for IgG and IgA. These sections showed no background staining and the negative control sections, as well as the sections from the normal skin were negative, therefore the findings can be regarded as specific. IgG (4), gamma-globulin (3), and C₃ (5) were found in the epidermal basal lamina and IgG was shown within stellate cells of the epidermis (6) in human cutaneous leishmaniasis. Complement, fibrinogen, and gamma-globulin were observed in the vessel walls and in the perivascular space in experimental cutaneous leishmaniasis of the guinea pig (10). All these findings might suggest that the necrosis of the skin and the ulceration in cutaneous leishmaniasis are immunologically induced phenomena. This theory was put forward by Bryceson *et al.* (15) who found cell necrosis in the prickle-cell layer followed by ulceration. Monroy *et al.* (16) agreed with this theory suggesting that the ulceration was brought about the same mechanism that caused the destruction of the macrophages.

Qualitative differences of the peroxidase positivity were noted among the lys⁺ macrophages and epithelioid cells: (a) not every cell of the mononuclear phagocytic system showed positive staining, (b) the immunoreactivity of the positive macrophages was diffuse and strong, and (c) the peroxidase-positive epithelioid cells and multinucleated giant cells contained finely granular and weaker reaction products. Similar findings have been reported in both acute and late stages of cutaneous leishmaniasis (6,7). It is known that during the maturation/activation process the monocyte undergoes various phases: monocyte - macrophages - epithelioid cell - multinucleated giant cell, showing characteristic morphological and biochemical features at every step (rev. 17). Immunohistochemistry revealed that well differentiated histiocytes were lys⁺, whereas the reactivity for this enzyme was absent in more de-differentiated cells (18). The macrophages in lepromatous

leprosy also show strong cytoplasmic lysozyme reactivity, but the epithelioid cells and giant cells of tuberculoid leprosy are negative (19). These latter findings are similar to a certain extent to our observations. Based upon these findings it can be assumed that the variability of immunoreactivity for lysozyme between the various cells of the mononuclear phagocytic system may be the expression of the different level of differentiation of these cells.

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IMMUNOHISTOCHEMICAL DEMONSTRATION OF S-100 PROTEIN ANTIGEN-CONTAINING CELLS IN CHRONIC CUTANEOUS LEISHMANIASIS

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Veress, B. & Hassan el A. M. Immunohistochemical demonstration of S-100 protein antigen-containing cells in chronic cutaneous leishmaniasis. *Acta path. microbiol. immunol. scand. Sect. A*, 93: 331-334, 1985.

The presence and distribution of S-100 protein antigen and lysozyme were investigated by the immunoperoxidase method in paraffin sections of 13 cases of chronic cutaneous leishmaniasis from Saudi Arabia. Varying numbers of S-100⁺ lys⁺ histiocytic reticulum cells were found in the dense inflammatory infiltrate in 11 out of 13 cases. These cells were considerably more numerous in the lesions dominated by cells of the mononuclear phagocytic system and granulomata than in the cases with plasma cellular or lymphocytic predominance. Activated lys⁺ macrophages, epithelioid cells, and multinucleated giant cells were always S-100⁺. Around the granulomata several S-100⁺ lys⁺ histiocytic reticulum cells could be found. These findings suggest that antigen-presenting cells are present in the inflammatory infiltrate of cutaneous leishmaniasis.

Key words: Leishmaniasis cutaneous; S-100 protein antigen; immunohistology.

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Histiocytes or macrophages belong to the mononuclear phagocytic system. The main task of these cells is to defend the host against intruders by phagocytosing and intracellular elimination and by stimulation of other types of cells to participate in the defence (Page *et al.* 1978, Pierce 1980, Lasser 1983). Recent immunological and histochemical investigations have revealed that the histiocyte population is heterogeneous, there being at least two subclasses of these cells: common inflammatory and antigen-presenting histiocytes (Lasser 1983, Selby *et al.* 1983, Watanabe *et al.* 1983a). The task of the former cells is phagocytosis and digestion, hence their name "scavenger" macrophages; whereas the latter ones take part in a complex immunological process. Dendritic and interdigitating reticulum cells of the lymph nodes,

Langerhans cells of the epidermis, and some of the histiocytes of the human intestine are considered to be antigen-presenting histiocytes (Takahashi *et al.* 1981, Lennert and Stein 1982, Selby *et al.* 1983).

Immunohistochemical methods by using specific antibodies against the enzyme lysozyme and the nerve-tissue specific antigen S-100 protein can differentiate between these two subclasses. The scavenger macrophages are S-200⁺ lys⁺; whereas the antigen-presenting histiocytes, called also T-zone histiocytes, are S-100⁺ lys⁺ (Takahashi *et al.* 1981, Watanabe *et al.* 1983a, b, Crocker and Hopkins 1984). In this paper we describe the presence of S-100⁺ lys⁺ histiocytic cells in the inflammatory infiltrate of 13 cases of chronic cutaneous leishmaniasis from Saudi Arabia and discuss their possible role.

MATERIALS AND METHODS

The patients were seen in the Dermatology Clinic, King Fahd Hospital, Hofuf, Saudi Arabia. There were 11 males and 2 females, aged from 3 to 45 years. They had not been treated and the lesions did not show any signs of healing. The duration of the disease was more than 1 year in each case. One half of a 4 mm punch biopsy was fixed in formaldehyde – mercuric chloride – glacial acetic acid fixative and embedded in paraffin. The other half of the material was processed for electron microscopy. Paraffin sections were stained with haematoxylin and eosin, Giemsa, and toluidin blue at pH 0.5. The parasite load was assessed in paraffin sections according to Ridley's method (1979).

Immunoperoxidase staining was carried out to localize S-100 protein- and lysozyme-containing cells with "Histoset" Immunoperoxidase Kit (Ortho Diagnostic Systems Inc., Raritan, N. J., U.S.A.). The last step of the staining procedure, the staining with Mayer's haemalaun, was omitted in parallel sections for easier evaluation of the positive reaction. In negative control sections from each case in each bath non-immune serum replaced the specific anti-S-100 or anti-lysozyme serum. Sections of human large intestine containing nerves, and tonsil served as positive controls in each bath of the staining. Five fields of each section were chosen at random from the diseased areas at $\times 500$ (objective lens: Zeiss Plan 40/0.65; eyepiece: Zeiss Kpl. W $\times 12.5$). The image of the field of view was projected on a white paper by an eyepiece mirror (Zeiss AG, West Germany) and the number of positively stained cells was counted. The total area of the 5 fields of view was 0.62 mm^2 .

RESULTS

The parasite load was 1+ or 2+ on the Ridley-scale. The inflammatory infiltrate consisted of cells of the mononuclear phagocytes (macrophages, epithelioid cells, and occasional multinucleated giant cells), lymphocytes, and plasma cells (Fig. 1). All the cases but two showed the presence of S-100⁺ cells. The numbers of these cells varied between 3 and 31 cells/5 HPF. The S-100⁺ cells were scattered within the dense inflammatory infiltrate; they were elongated and had fairly long, thin cell projections. Their size was 12–17 μm , the nuclei were large, vesicular, and contained distinct nucleoli (Fig. 2). A few smaller and round-shaped S-100⁺ cells were also seen. The comparison of identical areas of serial sections stained for S-100 and lysozyme, respectively, revealed that S-100⁺ cells were always lys⁺ and the lys⁺ macrophages contained no S-100 antigen. Often several S-100⁺ histiocytic cells could be observed around the granulomata (Fig. 3) but the epithelioid cells and multinucleated giant cells were S-100⁻. Langerhans cells of the epidermis and Schwann cells of the

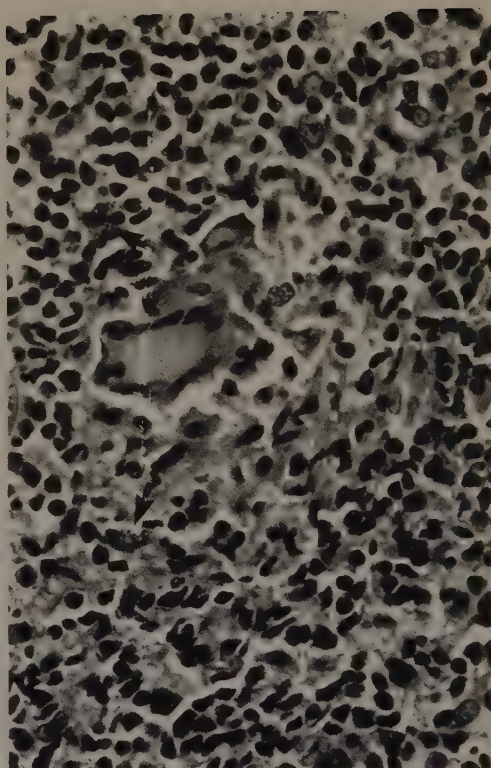


Fig. 1. The inflammatory infiltrate consists of lymphocytes, plasma cells, and cells of the mononuclear phagocytic system forming unorganized tubercle with a multinucleated giant cell. Arrows show histiocytes containing parasites. (Haematoxylin and eosin, $\times 180$).

peripheral nerves were always positive for S-100 in the sections. The S-100⁺ cells of the inflammatory infiltrate were negative after toluidin blue staining at pH 0.5, hence they were not mast cells.

It was interesting to note that 6 cases with the predominance of S-100⁺ lys⁺ macrophages or granulomata contained more than 7 S-100⁺ cells/5 HPF, with more than 20 S-100⁺ cells in four cases; whereas fewer than 5 S-100⁺ cells/5 HPF were found in all the other cases characterized by the predominance of plasma cells or lymphocytes, including two cases with the total absence of S-100⁺ histiocytic reticulum cells.

DISCUSSION

Recent immunological and immunohistochemical investigations have revealed strong expression of HLA-DR (Ia-like) antigens in some of the

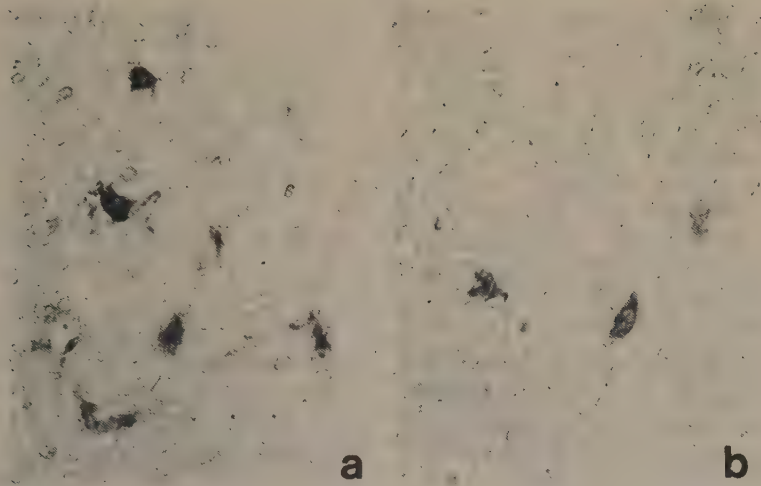


Fig. 2. S-100⁺ elongated cells with fine cell projections and vesicular nuclei in the inflammatory infiltrate from 2 cases. (Immunoperoxidase staining, x 100).



Fig. 3. The granuloma is surrounded by S-100⁺ histiocytic reticulum cells. The epithelioid cells and Langhans giant cells (arrows) are S-100⁻ (Immunoperoxidase staining, x 400).

histiocytic-type cells of the human intestine (Scott *et al.* 1980, Selby *et al.* 1983), in the epidermal Langerhans cells (Klareskog *et al.* 1977), and in the dendritic reticulum cells of the lymph nodes (Steinman *et al.* 1979, Lennert & Stein 1982). These cells, as well as the interdigitating reticulum cells of the thymus and lymph nodes, also contain the nerve-specific S-100 protein antigen in their cytoplasm (Cocchia *et al.* 1981, Takahashi *et al.* 1981, Nakajima *et al.* 1982), but they do not show positive staining for lysozyme, a common marker of the histiocytes (Watanabe *et al.* 1983b). It has been suggested that S-100⁺ lys⁺ dendritic reticulum cells recognize antigens and release mediators that stimulate T-cells (Steinman & Nussenzweig 1980), hence they have an important role in making the T-cell response more effective.

We found S-100⁺ lys⁺ histiocytic cells in the inflammatory infiltrate in 11 out of 13 cases of chronic cutaneous leishmaniasis. These cells had a relatively large, elongated body with long cell-projections resembling the dendritic and interdigitating reticulum cells of the lymph nodes. They were not so numerous as the IgG⁺ plasma cells or lys⁺ macrophages in these cases (Veress *et al.* in press). Nevertheless, the finding that the lesions with the predominance of histiocytes and granulomata contained considerably larger numbers of S-100⁺ histiocytic reticulum cells than the cases with plasma cell or lymphocyte predominance suggests that this is not a chance relationship. The localization of S-100⁺ lys⁺ histiocytic cells

around the epithelioid granulomata might also point to some kind of cooperation between these histiocytic reticulum cells and the cells of the granulomata. Similar observations have been reported between S-100⁺ cells and sarcoid tubercles by *Pineiro Maceira et al.* (1984).

Our findings suggest that antigen-presenting histiocytic reticulum cells are present in varying numbers of the inflammatory infiltrate of cutaneous leishmaniasis. The possible significance of this variation requires further studies of the disease, including that of the self-healing form.

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VASCULAR CHANGES IN HUMAN LEISHMANIASIS.

A light microscope and immunohistological study

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ABSTRACT

The study of human cutaneous leishmaniasis from Saudi Arabia (37 cases), and of Sudanese mucosal (52 cases) and visceral (27 cases) leishmaniasis revealed the occurrence of vascular alterations. Vasculitis with or without fibrinoid necrosis and fibrin thrombi were found both in the arteries and veins within the area of inflammation in cutaneous and mucosal leishmaniasis; whereas subendothelial oedema, hyalinosis, and intima proliferation were seen in the small arteries of the various organs in visceral leishmaniasis. Immunoperoxidase staining of the cutaneous leishmaniasis lesions showed the presence of IgG and IgA within the endothelial cells, in the media, and in the perivascular space of the diseased vessels. The formation of immune complexes, locally in cutaneous and mucosal leishmaniasis and circulating complexes in visceral leishmaniasis, is considered responsible for these vascular lesions.

INTRODUCTION

Leishmaniasis is a protozoal disease caused by an intracellular parasite living within the cells of the mononuclear phagocytic system. The disease occurs in three main forms: cutaneous, mucocutaneous, and visceral. The course and the outcome of the disease are influenced by both cell-mediated and humoral immune responses (Bryceson *et al.*, 1970; Howard *et al.*, 1982). Recent investigations have showed the presence of circulating immune complexes in human visceral and mucocutaneous leishmaniasis (Casali and Lambert, 1979; Pugin and Miescher, 1979; Desjeux *et al.*, 1980), and of *in situ* formed immune complexes in cutaneous leishmaniasis (Ridley and Ridley, 1984). Certain vascular changes are relatively common complications of immunologic disturbances. In the different forms of leishmaniasis a few papers have reported the occurrence of vascular alterations. The aim of the present paper is to describe the vascular changes found in cutaneous, mucosal, and visceral leishmaniasis of the Old World and to discuss the pathogenesis of these alterations in the light of immunohistological findings.

MATERIALS AND METHODS

The biopsy material from the lesions of 37 cases of cutaneous leishmaniasis from Saudi Arabia, and of 52 cases of Sudanese mucosal leishmaniasis, as well as the various organs of 27 cases of fatal visceral leishmaniasis in the Sudan were studied in the light microscope. The Sudanese material was fixed in 4% formaldehyde,

whereas the biopsies in Saudi Arabia were fixed in a formaldehyde - mercuric chloride - glacial acetic acid mixture. Paraffin embedded sections were stained with haematoxylin and eosin, Giemsa, PTAH, PAS, and Masson trichrome. Immunoperoxidase stainings for IgG, IgA, and IgM ("Histoset Kit", Ortho Diagnostic Systems Inc., Raritan, NJ, U.S.A.) were performed on paraffin sections of cutaneous leishmaniasis.

In negative parallel control sections from each case in each bath non-immune serum replaced the specific anti-Ig serum. Normal human skin from 5 persons was also used as a control for both positive and negative immunoperoxidase reactions. The vessels did not show any immunoreactivity in any of the control sections. Positive control sections of tonsil were included in each bath of staining for the control of the staining procedure.

RESULTS

Vascular alterations were found in 14 out of 37 cases of cutaneous leishmaniasis, in 22 out of 52 cases of mucosal leishmaniasis, and in 12 out of 27 cases of visceral leishmaniasis. In the cutaneous and mucosal forms of the disease the vascular changes were consisted of fibrinoid necrosis, vasculitis with neutrophils, lymphocytes, and mononuclear cells (Fig. 1), and occasionally hyalinosis. In six cases of mucosal leishmaniasis fibrin thrombi were also found. Both the arteries and veins were affected. Immunoperoxidase staining of cutaneous lesions revealed strong IgG and IgA positivity of some of the endothelial cells, of the media, and of the perivascular space (Fig. 2). In visceral leishmaniasis subendothelial oedema (Fig. 3), hyalinosis (Fig. 4), and intima proliferation were the common findings in the small arteries of the kidneys, liver, heart, retroperitoneum, and gastrointestinal tract.

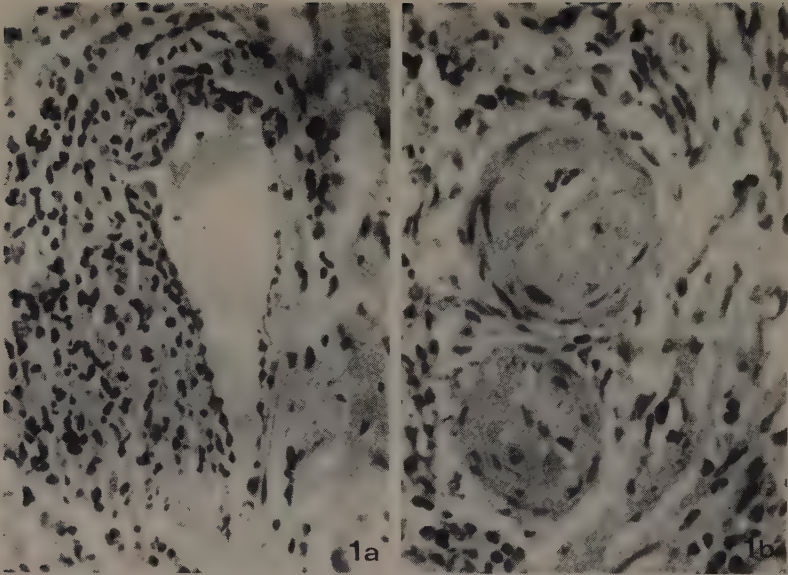


Fig. 1. Showing a/ vasculitis and b/ fibrinoid necrosis in a case of mucosal leishmaniasis. (Haematoxylin and eosin, a/ x180; b/ x220).

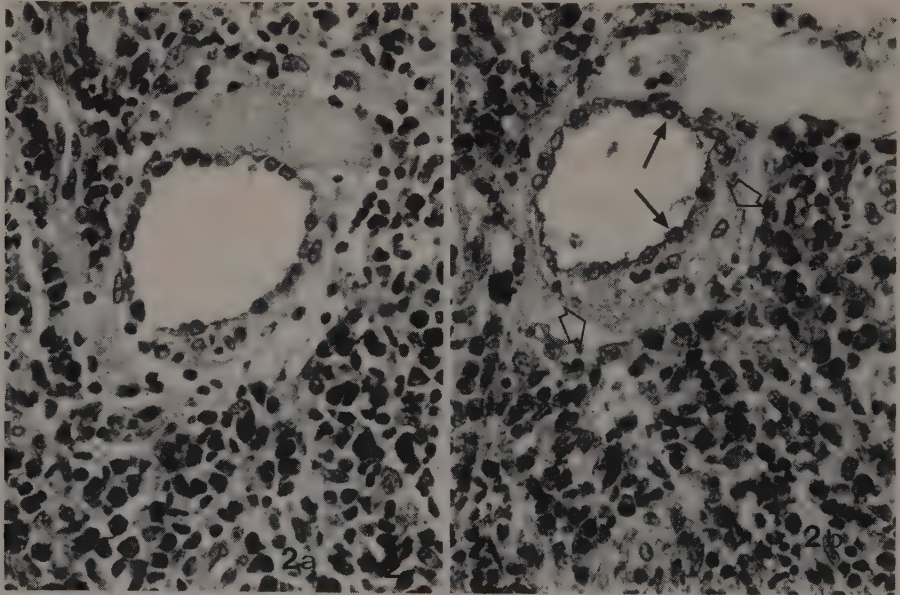


Fig. 2. a/ Haematoxylin and eosin stained section from a case of cutaneous leishmaniasis showing perivascular oedema and inflammatory cells around the vessel. (x260). b/ The same area of the section stained by immunoperoxidase method for IgG. The endothelial cells (arrows) and the oedematous perivascular space (open arrows) show positive staining. (C = collagen, x260).

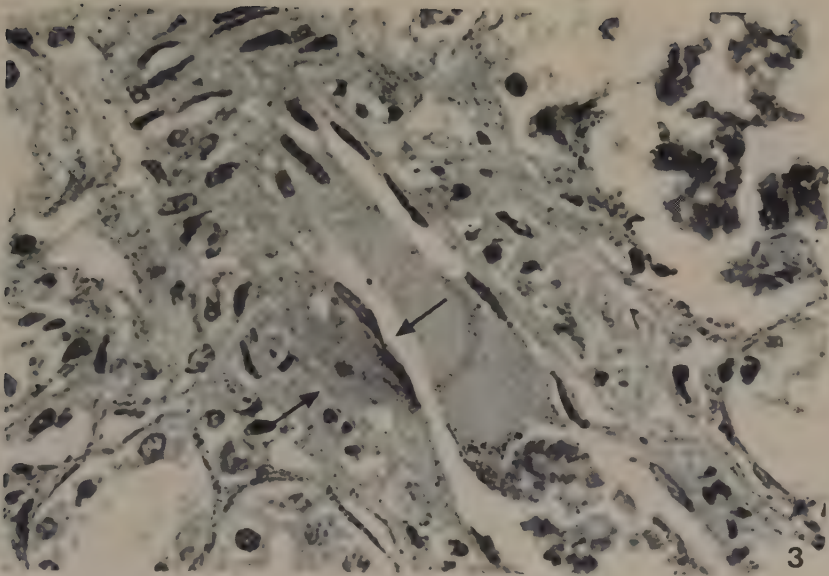


Fig. 3. Subendothelial oedema (between arrows) in the small renal artery from a kala-azar case. (Haematoxylin and eosin, x320).

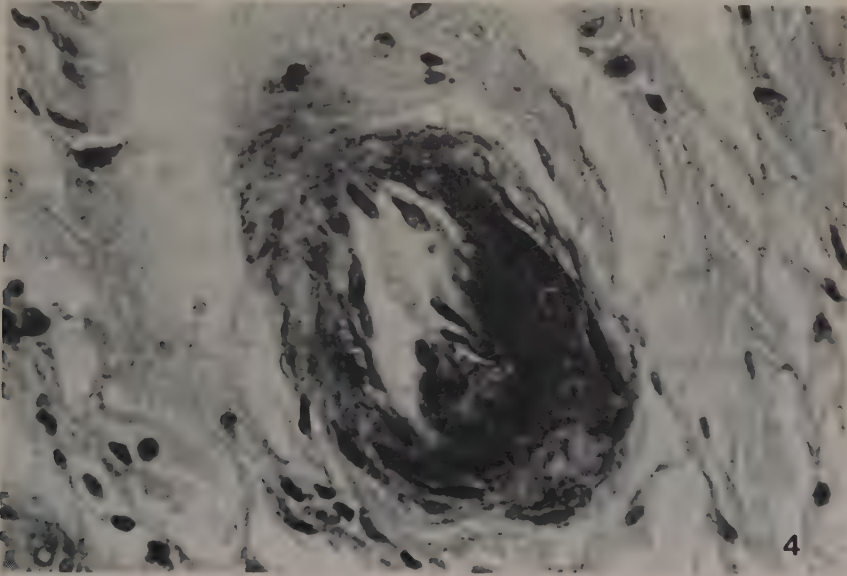


Fig. 4. PAS-positive hyalinosis of the small hepatic artery from kala-azar. (PAS, x 360).

DISCUSSION

There are a few papers in the leishmaniasis literature describing the vascular changes in the different forms of the disease. In cutaneous and mucocutaneous leishmaniasis of South America necrotising vasculitis with or without fibrin thrombi (Barbosa *et al.*, 1976; Morierarty *et al.*, 1978; Ridley *et al.*, 1980) and endarteritis obliterans (Klotz and Lindenberg, 1923) have been reported. In the most severe form of leishmaniasis, kala-azar with widespread visceral involvement, endothel proliferation has been observed (Andrade and Andrade, 1966). Diffuse intravascular coagulation in a case of kala-azar has been described as well (Blount *et al.*, 1980). In the lesions of experimental leishmaniasis endothel proliferation has been found in monkeys (Meleney, 1925) and in guinea pigs (Bryceson *et al.*, 1970), and acute vasculitis with fibrinoid necrosis has occurred in the skin lesions of mice and hamsters (Veress *et al.*, 1983).

It is known that circulating immune complexes can cause increased vascular permeability by damaging directly the endothelial cells (Mathews *et al.*, 1974; Poston and Davies, 1974) or by a more complex mechanism through complement binding (Roitt, 1980). Immune complexes can also cause the formation of fibrin thrombi in DIC (Robbin and Stetson, 1959).

Recent investigations have shown the presence of circulating immune complexes in the sera of patients suffering from visceral leishmaniasis (Casali and Lambert, 1979; Pugin and Miescher, 1979; Carvalho *et al.*, 1983; Pearson *et al.*, 1983) and mucocutaneous leishmaniasis (Desjeux *et al.*, 1980). Radwanski *et al.* (1974) have reported the perivascular presence of complement, fibrinogen, and gamma-globulin in the cutaneous lesions of guinea pigs and concluded that immune complexes were locally present in this localisation. Ridley and Ridley (1984) have shown the *in situ* formation of immune complexes in human cutaneous leishmaniasis and suggested that immune complexes stimulated the formation of tuberculoid granulomas.

The vascular changes observed locally in cutaneous and mucosal leishmaniasis and in several organs in the fatal visceral leishmaniasis cases of our material were composed of the various manifestations of increased vascular permeability (subendothelial oedema, fibrinoid necrosis, hyalinosis), vasculitis, and the formation of fibrin thrombi. We could also show the presence of IgG and IgA in the endothelial cells, in the media, and in the perivascular space. These latter findings could mean that immune complexes are deposited in these localisations. It is known that the outcome of the formation of immune complexes depends on the absolute and

the relative proportions of antigen and antibody (Roitt, 1980). Therefore, our findings suggest that the local vasculitis and thrombosis in cutaneous and mucosal leishmaniasis are the sequel of a local immunological reaction due to antibody-excess and the vascular lesions in visceral leishmaniasis can be the result of the formation of circulating immune complexes caused by excess amount of antigen.

ACKNOWLEDGEMENTS

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The Ultrastructural Morphology of Human Cutaneous Leishmaniasis of Low Parasite Load

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El Hassan AM, Veress B, Kutty MK. The ultrastructural morphology of human cutaneous leishmaniasis of low parasite load. Acta Derm Venereol (Stockh) 1984; 64: 501-505.

The ultrastructural morphology of the inflammatory infiltrate in cutaneous leishmaniasis with low parasite load from seven patients was studied. Cells of the mononuclear phagocyte system (macrophages and epithelioid cells), plasma cells, and lymphocytes formed the infiltrate. Disseminated shrinkage necrosis or apoptotic as well as coagulative necrosis with loss of the plasma membrane of single macrophages occurred. An intimate contact was observed between macrophages and lymphocytes. The relationship of the morphological alterations to the persistence of the chronic inflammatory reaction and the possible explanations of the macrophage necrosis are discussed. *Key words: Macrophage; Necrosis.* (Received January 16, 1984.)

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Leishmania are obligate intracellular protozoa, that reside in macrophages. Depending on the species of the organism three forms of the disease occur in man: visceral, mucocutaneous, and cutaneous leishmaniasis. The latter is caused by different strains in different geographical regions. In the Middle East *L. major* produces the classical oriental sore and a variety of other clinical forms including mucocutaneous leishmaniasis (1). The lesion heals spontaneously or with treatment but in some patients it persists for many months or years and may become refractory to treatment. This is particularly true for the recidiva form which shows signs of central healing but recurs and spreads at the margin (2). The diffuse form of the disease which is described in Ethiopia and South America produces a non-healing lesion that continues for years and shows many parasites.

The reasons for the persistence of the infection in some cases are poorly understood. Cell-mediated immunity appears to be important for the elimination of the parasite (3) but it appears that the excessive cell-mediated reactivity is responsible in some way for the persistence of the recidiva form. This preliminary communication describes the occurrence of apoptosis, an immunologically induced cell necrosis (4) in chronic cutaneous leishmaniasis that failed to heal despite a low parasite load. It is proposed that persistence of leishmanial antigen in the macrophages or macrophage plasma membrane receptors cross-reacting with leishmanial antigen can prolong the inflammatory reaction and delay healing.

PATIENTS AND METHODS

Patients were seen in Hufuf oasis in the Eastern Province of the Kingdom of Saudi Arabia. There were six males and one female, aged between 3 and 45 years. They had no treatment and showed no signs of healing. 4 mm punch biopsy of the lesions was performed under local anaesthesia. One half was fixed in a formaldehyde mercuric chloride-acetic acid mixture. Paraffin sections were stained with haematoxylin and eosin, and Giemsa and examined for parasite load and cellular reaction. Smears from the cut surface of the fresh biopsy were fixed in methanol and stained with Giemsa for

parasites. The material for electron microscopy was fixed in 4% glutaraldehyde, 4°C for one hour, washed in phosphate buffer pH 7.2, postfixed in 1% OsO₄ and embedded in Epon. Sections were cut on an LKB Ultratome III. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined in a Zeiss EM-9 electron microscope. The electron micrographs were taken on Gevaert Scientia 23D56 P3AH films. 1 µm sections were stained with toluidine blue for light microscopy.

RESULTS

The parasite load was 1+ or 2+ on the Ridley-scale (2), in which the maximum was 5+ representing a heavy load of amastigotes in all parts of the section.

The composition of the inflammatory infiltrate was basically similar in all cases examined. 1 µm thick sections showed very few parasitized histiocytes in one case only, whereas no parasites were observed in the thick sections of the other 6 cases though some parasites were always seen in the paraffin sections or smears. The inflammatory infiltrate was composed of cells of the mononuclear phagocyte system, plasma cells, and lymphocytes. Intermingled with these cells there were scattered, small, very dark, compressed cells without clearly defined nuclei (Fig. 1).

Electron microscopy revealed that the cells of the mononuclear phagocyte system were activated macrophages and epithelioid cells. The former ones were smaller and elongated cells, their cytoplasm had long, slender projections and the hyaloplasm was electron dense, darker than that of the epithelioid cells. These activated macrophages also contained numerous cytoplasmic vacuoles. The large, polygonal and pale epithelioid cells had bean-shaped nuclei with finely distributed chromatin. Numerous mitochondria, polyribosomes, and lysosomes were a constant feature of these cells (Fig. 2). Several lymphocytes showed the signs of activation: they were larger, had short cytoplasmic projections and an increased number of mitochondria and endoplasmic reticulum. A close contact between the plasma membrane of the lymphocytes and the macrophages was a common finding.

The scattered, small, very dark cells showed a condensed, very electron dense cytoplasm which occasionally showed shadows of the cell organelles. The nuclei were pyknotic with marked condensation of the chromatin and sometimes the occurrence of paler, irregular areas within the condensed chromatin (Fig. 3). Other macrophages showed the signs of coagulative necrosis: oedematous degeneration or extensive vacuolization of the cytoplasm, fragmentation or dissolution of the nuclei, and partial or complete loss of the plasma membrane.

DISCUSSION

The mechanisms of parasite elimination and the persistence of the inflammatory process, hence the clinical disease, despite a low parasite load are still poorly understood. It was believed that the most important mechanism for the elimination of the parasites was necrosis of the parasite-loaded macrophages and not the development of delayed hypersensitivity (2, 5). Another possible mechanism, at least in some geographical areas, may involve lymphokine activated macrophages. Veress et al. (6) have shown that parasites were being destroyed in the cytoplasm of activated macrophage in patients with mucosal leishmaniasis. Intracellular digestion of *Leishmania* parasites and their elimination was described in connection with the development of skin-test hypersensitivity of the delayed type (7).

Two cell types of the mononuclear phagocyte system were seen in the inflammatory infiltrate of low parasite load in our cases. One was the activated macrophage with many cytoplasmic vesicles, long cell projections, and well developed endoplasmic reticulum. The other one was the epithelioid cell with very abundant, pale cytoplasm. These cells

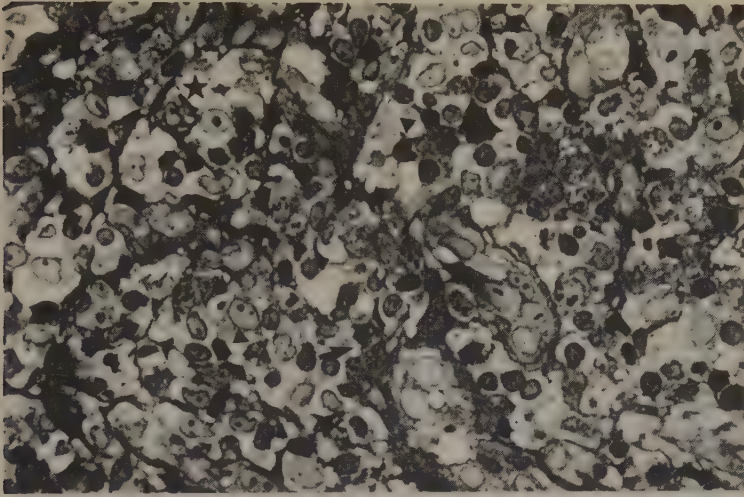


Fig. 1. Densely packed infiltrate with large, pale epithelioid cells (star) and darker macrophages (arrow) as well as shrunken, compressed cells without well defined nuclei (arrowheads). $\times 160$.

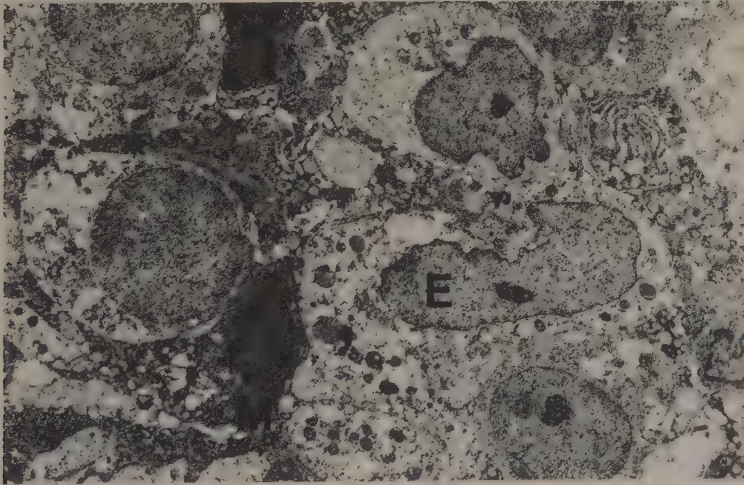


Fig. 2. Showing large epithelioid cells (E) and macrophages (M). $\times 1800$.

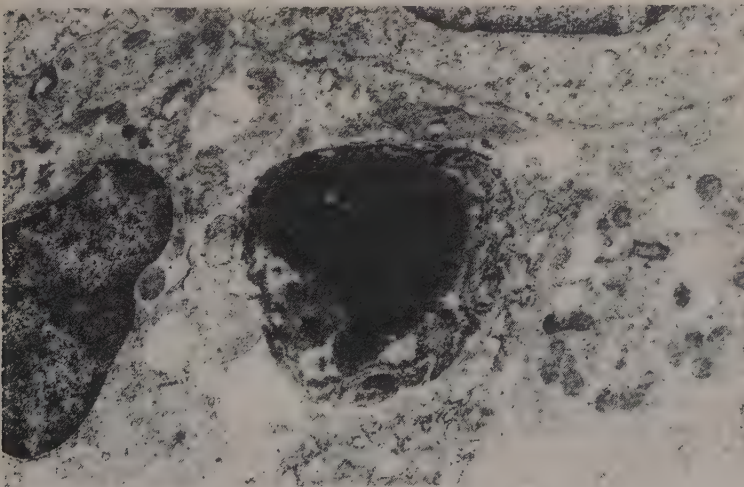


Fig. 3. Apoptotic body with condensated, electron dense chromatin, irregular electron lucent areas, and dark cytoplasm without recognizable organelles. $\times 4900$.

represent different stages of macrophage development with different functional activities as was shown in experimental models (8, 9, 10). We could find in these cases a special type of cell necrosis: the necrotized cells showed a marked condensation of the nuclear chromatin and that of the cytoplasm. These cells eventually disintegrated into smaller fragments consisting of cytoplasm and nuclear material, possibly appearing as haematoxyphil bodies in the light microscope, a fairly common finding in cutaneous leishmaniasis (2). These features of cell necrosis are those of apoptosis (4) which is caused by killer T-lymphocytes as was shown in vitro experiments (11). Apart from apoptosis, coagulative necrosis with the loss of plasma membrane was also seen in our cases. Bryceson et al. (12) proposed that the loss of plasma membrane of the necrotizing macrophages in experimental leishmaniasis was due to immunological mechanism.

In our cases there was an intimate contact observed between activated lymphocytes and macrophages. It is known that leishmanial antigen can be present in the macrophages (12, 13, 14, 15). The antigen presentation is thought to be essential in the activation of lymphocytes (16). On the other hand, antigen-carrying macrophages or cells which have membrane receptors cross-reacting with leishmanial antigen, as shown on the plasma membrane of human red blood cells (17), can be the target cells of activated killer lymphocytes or specific antibodies. Hence, one can speculate that in these cases of chronic cutaneous leishmaniasis of low parasite load, when the treatment with antileishmanial drugs is often unsatisfactory (El Hassan, personal observation), the persistence of defected macrophages having either leishmanial antigen or cross-reacting membrane receptors, and not the parasites themselves are responsible for unsuccessful treatment. It is interesting in this connection to cite two publications reporting the healing of persistent cutaneous leishmaniasis after the application of transfer factor (18) or BCG and cord factor (19) which stimulate the host's immune response but not antileishmanial drugs.

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Electron microscope investigations on leishmaniasis in the Sudan

I. Morphometric studies on *Leishmania* amastigotes in various forms of human leishmaniasis

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Statistical-morphometric analysis of certain ultrastructural features of amastigotes, such as their diameter, contour length and microtubule number demonstrated that the *Leishmania* parasites found in seven cases of human mucosal, cutaneous and visceral leishmaniasis in the Sudan belonged to the same group of the genus *Leishmania*. The values of these measurements corresponded to those described in *L. donovani*.

In the Sudan leishmaniasis is mainly visceral leishmaniasis (VL), but in some regions of the country cutaneous (CL) and a special, exclusively mucosal (ML), form also exists (Hoogstraal and Heyneman, 1969; Milosev *et al.*, 1969; Abdalla *et al.*, 1973). In spite of the still missing unequivocal evidence, *L. donovani* has been suggested as the only causative organism for all clinical types of leishmaniasis in the Sudan (Milosev *et al.*, 1969), although it may be that in certain provinces *L. tropica* can cause skin lesions (Abdalla *et al.*, 1973).

Recent morphological examination of different strains of *Leishmania* (Gardener *et al.*, 1977) have shown that certain parameters (size, circumference, number of subpellicular microtubules) differ significantly between the various species, so that such examinations can help in species determination. On the basis of these results it seemed useful to analyse the electron micrographs of *Leishmania* amastigotes obtained from human mucosal, cutaneous and visceral leishmaniasis. The present communication describes our results and concludes that the parasites found in these cases of leishmaniasis in the Sudan all belong to the same species of *Leishmania*.

MATERIALS AND METHODS

Biopsy specimens from the lesions of three cases of ML and two cases of CL were cut into 1 mm cubes and fixed in cold 4% formaldehyde in phosphate buffer pH 7.4, isotonized by sucrose for four hours. Postfixation was carried out in 1% OsO₄ in Millonig's buffer solution at +4°C for one hour prior to embedding in Durcupan ACM (Fluka AG, Switzerland). Ultrathin sections were cut with an LKB III Ultratome, stained with uranyl acetate and lead nitrate and then examined with a JEOL 100B (JEOL, Japan) electron microscope at the 2nd Central Laboratory for Electron Microscopy, Semmelweis Medical University, Budapest, Hungary. Micrographs were taken on Gevaert 23 D50 plates.

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The splenic punctures from two patients suffering from VL were homogenized in 10 ml Hank's solution and the homogenate injected intraperitoneally into three hamsters. The animals were killed with ether four weeks later, and small cubes of the spleens processed for electron microscopy as before.

For morphometric studies only, parasites cut approximately perpendicularly to the long axis (including part of the nucleus) were considered (Fig. 1). The diameters of the parasites and their contour lengths were determined by means of a map measurer from photographs at a standard magnification of $\times 90\,000$ obtained after enlargement of the original negatives. The sizes of the individual microtubules (both the longer and shorter diameters), as well as the distances between two neighbouring microtubules were measured directly on photographs after magnification to $\times 180\,000$. A total of 123 parasites was examined from ML, 56 from CL and 105 from VL.

In the electron micrographs transversally cut microtubules could be found in only about 25–35% of the total contour length (Fig. 1). Hence, the number of these sub-pellicular tubules could be counted only in these regions. In each the length of this region and the number of microtubules in it was determined directly and from these data the total microtubule count in each parasite was obtained as follows:

$$\text{total microtubule count} = \text{counted number} \times \frac{\text{total contour length}}{\text{measured region}}.$$

All results were statistically evaluated including two-sided *t*-test for significance (Armitage, 1971). The results are shown in Dice-Leraas type diagrams (Dice and Leraas, 1936), in which the vertical lines represent the mean, the horizontal lines represent the extreme values of the range, the black regions indicate the standard deviation and the white rectangles indicate twice the standard error.

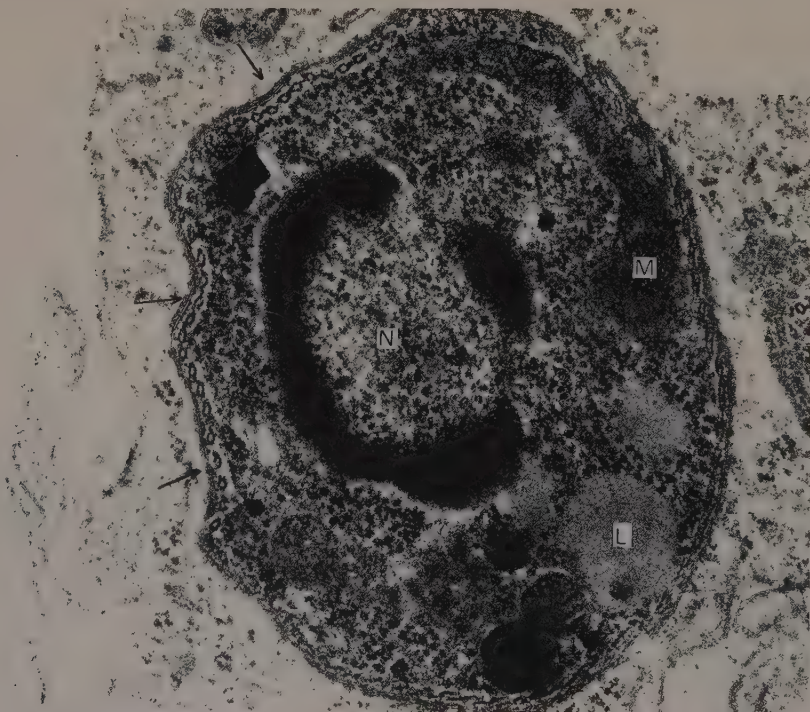


Fig. 1. Showing a parasite with part of the nucleus (N), mitochondrion (M) and lipid droplet (L). Microtubules cut transversally (arrows) could not be observed along the whole circumference. ($\times 42\,000$, lead nitrate-uranyl acetate).

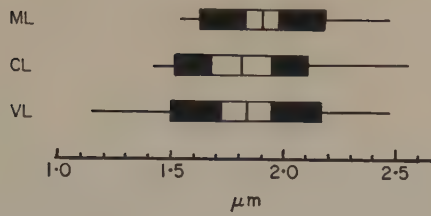


Fig. 2. Dice-Leraas diagram of section diameter of the parasites.

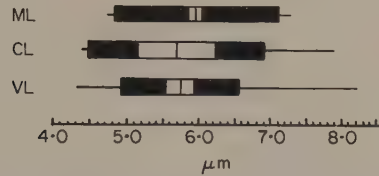


Fig. 3. Dice-Leraas diagram of section contour length of the parasites.

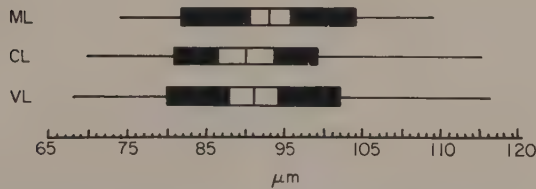


Fig. 4. Dice-Leraas diagram of microtubule number.

TABLE 1

*Showing epidemiological data and site of the lesions.
All seven patients were male*

<i>Type of leishmaniasis</i>	<i>Age</i>	<i>Place of residence (and province)</i>	<i>Site affected</i>
Mucosal	36	Gedaref (Kassala)	nose
	43	El Fasher (Darfur)	oral cavity
	31	Gedaref (Kassala)	larynx
Cutaneous	24	Gedaref (Kassala)	arms
	35	Wad Medani (Khartoum)	face
Visceral	22	Juba (Equatoria)	viscera
	17	Kassala (Kassala)	viscera

RESULTS

The significant clinical and epidemiological features of the seven biopsied patients are listed in Table 1.

The results of the measurements and their statistical analysis are given in Fig. 2 for parasite diameter, in Fig. 3 for section contour length and in Fig. 4 for microtubule number. In these diagrams the white rectangles overlap and corresponding to this the two-sided *t*-test revealed no significant differences in these parameters between the three strains (Table 2). Preliminary morphometric study of microtubule sizes and intervals did not show any significant variation between the parasites of the three strains.

TABLE 2
Summary of measurements

Group	Sample number	Mean diameter	Mean contour length	Microtubule number
ML	213	$1.91 \pm 0.04 \mu\text{m}$	$5.99 \pm 0.05 \mu\text{m}$	93 ± 1.4
CL	56	$1.82 \pm 0.07 \mu\text{m}$	$5.72 \pm 0.29 \mu\text{m}$	90 ± 1.8
VL	105	$1.84 \pm 0.06 \mu\text{m}$	$5.76 \pm 0.11 \mu\text{m}$	91 ± 1.7

Differences in means between groups are not significant ($P > 0.5$).

DISCUSSION

The various species of *Leishmania*, as well as their amastigote and promastigote forms, show similar ultrastructure (Bray, 1974; Bourland-Reinert and Nicolay, 1975; Kanan, 1975; Poulter, 1976; Sandbank, 1976; Hentzer and Kobayashi, 1977). At present, enzymic and serological variations are recognized (Schnur *et al.*, 1972; Gardener *et al.*, 1974; Matossian-Rogers *et al.*, 1976; Schnur and Zuckerman, 1977), but the only morphological difference between the various species seems to be the variation in size of the parasites and in the number of their subpellicular microtubules. Gardener *et al.* (1977) summarized data from the literature on the sizes of various species of *Leishmania*. From this review it is clear that *L. donovani* is the smallest of all. A survey of the literature also shows that *L. donovani* has the smallest microtubule count, found to be 120–220 in *L. enriettii* (Kanan, 1975), 130–220 in *L. mexicana* (Garnham and Bird, 1962; Creemers and Jadin, 1967), 125 in *L. mexicana amazonensis* (Gardener, 1974), 90–94 (Bourland-Reinert and Nicolay, 1975; Hentzer and Kobayashi, 1977) or 80–125 (Angelopoulos, 1970) in *L. tropica* and 80–120 in *L. donovani* (Sanyal and Sen Gupta, 1967). None of these numbers was obtained as a result of statistical analysis. However, in 1977 Gardener *et al.* showed in a statistical-morphometric study that the means of the microtubule counts in two isolates of *L. donovani* were 77 and 81, whereas those of two strains of *L. mexicana* were 118 and 114.

Our own statistical analysis shows that the mean size of the parasites found directly in human lesions of ML and CL and in spleens of hamsters inoculated by splenic puncture material from cases of human VL, as well as the means of the microtubule counts, does not vary significantly (Table 2). Hence one can conclude that the parasites may belong to the same species. Furthermore, these values conform closely to those found by Gardener *et al.* (1977) for *L. donovani*, suggesting that the parasites which cause the three forms of human leishmaniasis in the Sudan might well all be *L. donovani*. We agree completely with the opinion of these authors that only statistical analysis can serve as a basis to differentiate

the various species of *Leishmania*, especially, because of its wide range, with regard to the microtubule count (the range was 58–118 in the study of Gardener *et al.* and 68–116 in our own material).

Our results on the types of parasites recovered from the three forms of Sudanese leishmaniasis agree well with both early and recent serological typing of the various Sudanese strains. Adler *et al.* (1966) found antigenic similarity between strains isolated from human CL and VL, as well as from different animals, and concluded that these parasites all belonged to one species. Recently, Chance *et al.* (1978) showed by biochemical and serological methods that 15 leishmanial strains isolated from human VL and CL, from rodents, carnivores and *P. orientalis* in the Sudan were identical with *L. donovani* *s.l.* The features of one Sudanese strain isolated from a case of CL from Darfur province, however, corresponded to *L. major*. It is worth stressing that the Sudan is the only country in the Aethiopian zoogeographical region where cases of CL caused by *L. donovani* have occurred. As to which form of human leishmaniasis develops in the Sudan, one might speculate that this depends on the resistance of the host, and especially on the state of the immune defences. In this connection, it is useful to recall that in all cases of fatal VL in the Sudan examined at autopsy there was a marked depletion of thymus-dependent lymphoid regions (Veress *et al.*, 1977). The question of parasite, disease form and immunological status seems worth further investigation.

From the pattern of the subpellicular tubules within one section of the parasite one could deduce a helical arrangement, as was shown by Angelopoulos (1970) in specially prepared protozoons of the family Trypanosomatidae. This author also described individual differences between the parasites of the same species. Gardener *et al.* (1977) found tighter and looser spiralling of the microtubules in various species of *Leishmania*: in *L. donovani* isolated in Ethiopia and Liverpool transversally cut microtubules were found along the whole contour length of the parasites, pointing to a looser helix, while in *L. enriettii* all sections of the protozoons contained transversally and longitudinally cut microtubules, suggesting a tight helix. In our material nearly all the parasites had both transversally and longitudinally cut microtubules. The slightly higher number of microtubules and the difference in arrangement from the *L. donovani* strains studied by Gardener *et al.* (1977) might suggest that the parasites found in the present seven cases of Sudanese ML, CL and VL are a subgroup of *L. donovani*.

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Electron microscope investigations on leishmaniasis in the Sudan: II

Ultrastructural morphology of macrophage-parasite interaction in human and hamster macrophages *in vivo*

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An electron microscope study was made to compare the details of ultrastructural morphology of parasite-macrophage interaction in Sudanese human mucosal leishmaniasis and hamster visceral leishmaniasis produced by *Leishmania* amastigotes obtained from human mucosal lesions.

There were some differences between human and animal infections. In parasitized human macrophages there were a few, little or no parasitophorous vacuoles; no membranous cytoplasmic degeneration and no dividing amastigotes but ultrastructural signs of enhanced secretory activity. By contrast, in hamster splenic macrophages many large parasitophorous vacuoles developed around the parasites, occasionally dividing amastigotes were seen and there was marked membranous degeneration of macrophages without signs of enhanced secretory activity.

These observations suggest certain difference in macrophage-parasite interaction between *in vivo* and *in vitro* conditions, as well as between human and animal infections.

Leishmaniasis is a parasitic disease caused by different types of *Leishmania*, an obligate intracellular parasite. The course of infection in leishmaniasis depends mainly on the macrophage-parasite relationship because macrophages are the only host cells of these parasites and considered an important part of the immune system. These cells are able to internalize and destroy microorganisms, secrete various substances and take part in antigen-presentation (Calderon and Unanue, 1974; Unanue, 1976; Page *et al.*, 1978).

Recently, some work has been done on the ultrastructural characteristics of the macrophage-*Leishmania* interaction in *in vitro* experiments (Akiyama and McQuillen, 1972; Alexander and Vickerman, 1975; Lewis and Peters, 1977; Chang and Dwyer, 1976, 1978; Ebert *et al.*, 1976, 1979). In this paper we describe and compare the details of the macrophage-parasite relationship as seen in Sudanese mucosal leishmaniasis and hamsters infected by parasites obtained from human mucosal lesions.

MATERIALS AND METHODS

Biopsy specimens from the lesions of three cases of mucosal leishmaniasis were divided into two parts: one half was homogenized in 10 ml Hank's solution and the homogenate was injected intraperitoneally into three hamsters. The animals were killed after 15 weeks and 1 cm³ cubes of the spleen were fixed in cold 4% formaldehyde in phosphate buffer (pH 7.4) isotonized by sucrose, for four hours. Postfixation was performed in 1% OsO₄ in Millonig's buffer solution at 4°C for one hour prior to embedding in Durcupan ACM (Fluka AG., Switzerland). Ultrathin sections were cut with an LKB III ultratome, stained with uranyl acetate and lead nitrate and then examined with a JEOL 100 B electron microscope at the 2nd Central Laboratory for Electron Microscopy, Semmelweis Medical University, Budapest, Hungary. Micrographs were taken on Gevaert 23 D50 plates.

The other half of the human biopsy specimen was cut into 1 cm³ cubes in each case and processed for electron microscopy as described above.

RESULTS

Observations on Macrophages in Human Mucosal Leishmaniasis

Only intracellular *Leishmania* parasites could be found in all three cases. The amastigotes were surrounded by a unit membrane and all parasites had this 'host membrane' (Fig. 1). Between the pellicular and 'host' membranes there was a 3 nm thick electron lucent space. In a few macrophages this space was widened and contained an accumulated finely granular material, but no large parasitophorous vacuoles (PV) containing more amastigotes were seen in any of the macrophages. Nevertheless, there were numerous disorganized, partly lysed amastigotes in the host cells.

The cytoplasmic organelles of the macrophages showed signs of an enhanced synthetic activity: the tubules of the rough surfaced endoplasmic reticulum were dilated and contained a moderately dense material (Figs. 1, 2). The Golgi apparatus was very well developed, with dilated central tubules and many peripheral vesicles. There were numerous primary lysosomes and the mitochondria did not show any degenerative changes (Figs. 1, 2). Coated vesicles up to 90 nm in diameter were seen close to or fused with the plasma membrane in many macrophages (Fig. 2). Sometimes fine cytoplasmic filaments were seen near these vesicles. Neither membranous degeneration nor necrotized macrophages were found in the tissue blocks examined.

Observations on Splenic Macrophages of Hamsters

Leishmania organisms were found both intra- and extracellularly. Some extracellular parasites were surrounded by thin cytoplasmic projections of macrophages devoid of cell organelles but no macrophage was ever found with a disrupted plasma membrane in connection with internalization of the micro-organisms. Most internalized amastigotes were found in PVs (Fig. 3) containing granular material and disarranged membranes, apart from the parasites. Sometimes the membrane of a very large PV seemed discontinuous, without any sharp boundary towards the host cell's cytoplasm, but there were many degenerative membranous bodies in close vicinity to a PV (Fig. 4). Occasional enlarged parasites had two rudimentary flagella. The ultrastructure of nearly all the parasites was intact; their cytoplasm contained many phagosomes and in the flagellar pouches membrane-bound vesicles.

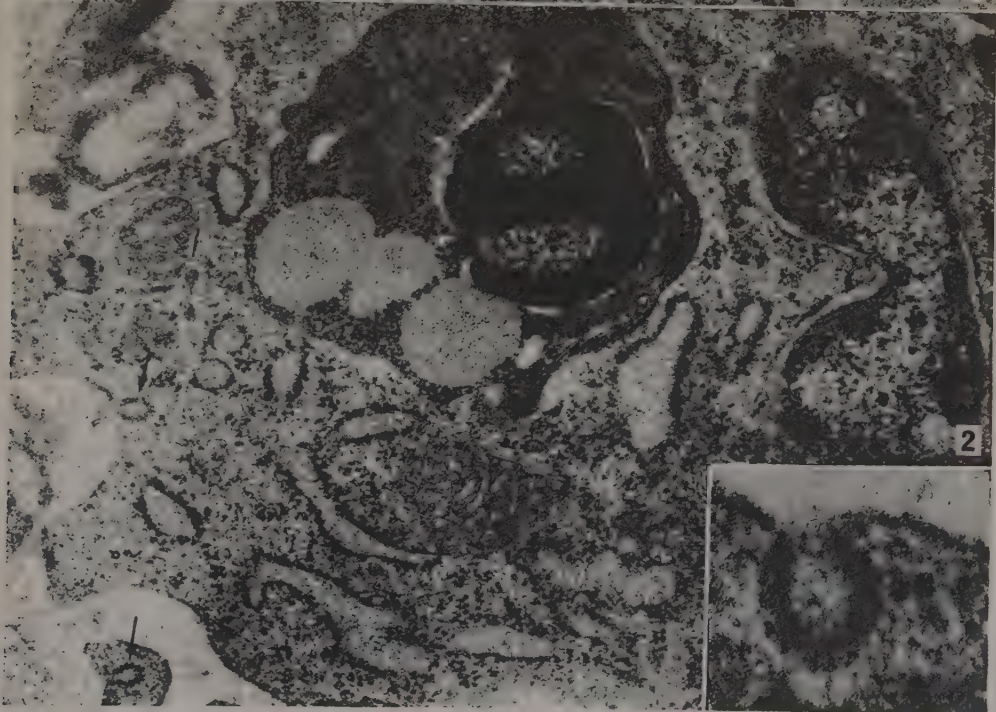
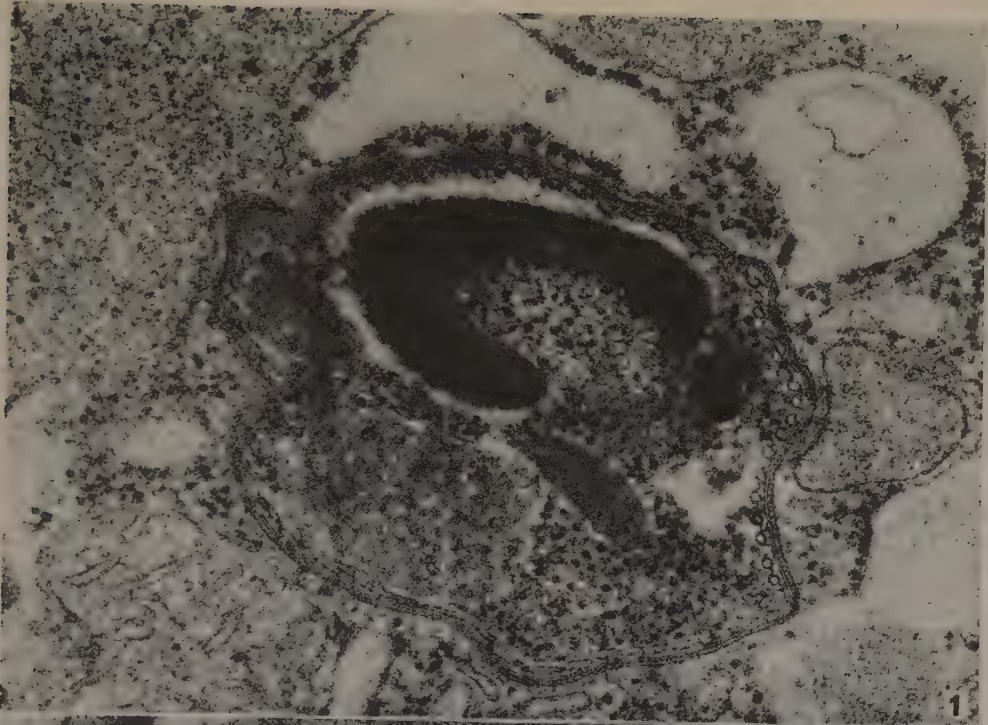


Fig. 1. Detail of a macrophage from human mucosal leishmaniasis. The *Leishmania* amastigote is tightly surrounded by 'host membrane'. In the cell's cytoplasm there are dilated cisternae of rough-surfaced endoplasmic reticulum and a mitochondrion. $\times 30\,000$.

Fig. 2. Detail of a macrophage from human mucosal leishmaniasis showing dilated endoplasmic reticulum filled with a granular material. Coated vesicles are fused with the plasma membrane (arrows). $\times 15\,000$. Inset: fused coated vesicle at high magnification $\times 75\,000$.

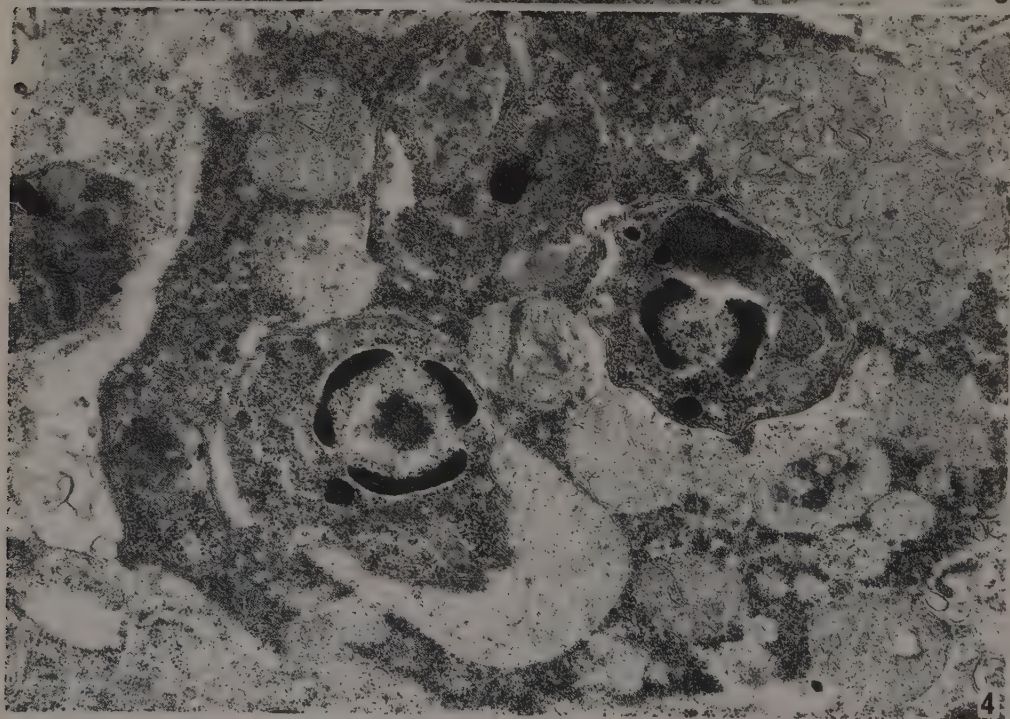


Fig. 3. Detail of a macrophage from hamster spleen. *Leishmania* amastigote lies in a large parasitophorous vacuole. The vacuole's membrane is continuous with that of the 'host membrane' (arrows). The tubules of the endoplasmic reticulum are not dilated. $\times 18\,000$.

Fig. 4. Detail of a macrophage from hamster spleen showing marked membranous degeneration of the host cell with three undamaged amastigotes. $\times 9\,000$.

Macrophages without a PV showed similar features of cellular activity to those seen in human mucosal leishmaniasis. Nevertheless, many macrophages revealed various signs of advanced degeneration. The hyaloplasm of these cells was very dense, the mitochondria were shrunken with an electron-dense matrix, and there was no well organized Golgi apparatus or endoplasmic reticulum. Instead, there were numerous small, highly osmophilic granules, larger less electron-dense areas and membranous, degenerative bodies (Fig. 4).

DISCUSSION

Mitchell (1979), in his review on host-protective immunities to parasites, concluded that there are three main areas where the protective immunity can be influenced: parasite-antigenicity, intramacrophage environment and host immune-responses. The macrophage-*Leishmania* parasite system offers a useful model for the study of the macrophage's response to parasite invasion, including the morphological changes of the host cells. We have attempted to compare the ultrastructural features of parasitized macrophages in human mucosal leishmaniasis and in the spleen of hamsters infected with parasites obtained from these human lesions. There were striking differences between the human and hamster macrophages (Table); these might be an expression of a different macrophage-parasite interaction and could be related to parasite survival.

TABLE

Summarizing differences in macrophage-ultrastructure

	<i>Human mucosal leishmaniasis</i>	<i>Hamster visceral leishmaniasis</i>
Parasitophorous vacuole	few/no little	many/large
Necrotized parasites	often	seldom
Coated vesicles	often/many	seldom/few
Membranous degeneration	no	yes
Dividing amastigotes	no	yes

The morphological sign of this survival could be the presence or absence of a PV. The first step in the development of these vacuoles is the accumulation of a finely granular material between the pellicular and 'host' membranes as has been shown in both *in vitro* studies (Alexander and Vickerman, 1975; Lewis and Peters, 1977; Chang and Dwyer, 1978; Ebert *et al.*, 1979) and our own *in vivo* observations. Recent ultrahistochemical studies have also found that the macrophage lysosomes can fuse with the PV and their content of hydrolytic enzymes reaches the lumen of the vacuole without apparently damaging the parasites (Alexander and Vickerman, 1975; Chang and Dwyer, 1976, 1978; Lewis and Peters, 1977; Ebert *et al.*, 1976, 1978; Sandbank and Ben David, 1979). In our material, PVs were never demonstrated in human parasitized macrophages, while these structures were common in hamster macrophage cells. At the same time, we could hardly find any degenerating parasites in the large PVs of hamster macrophages. Similar findings were described in cultured and infected macrophages by Lewis and Peters (1977) and Alexander and Vickerman (1975). In human dermal leishmanoid and cutaneous leishmaniasis no large PVs were seen by any workers (Sanyal and Sen Gupta, 1967; Pham *et al.*, 1970; Hentzer and Kobayashi, 1977), and Sandbank and Ben David (1979) found many degenerated parasites surrounded by a tight 'host membrane' in the cytoplasm of the macrophages—as we did. A possible explanation of the development of a

large PV, and the difference between human and hamster macrophages seen in our material, might be that the PV results from the inability of the macrophage to kill the parasite: lysosomes fuse with the PV but their enzymes cannot damage the amastigote, hence the vacuole increases in size.

We believe that the limiting membrane of the PV ruptures at a certain stage and that the host's lysosomal or the parasite's enzymes (or both) can reach the cytoplasm and damage the host cell. The membranous structures, which were a common finding in hamster macrophages but never occurred in human cells, can be formed by the decomposed lipoproteins of the damaged cell organelles. Similar membranous bodies have also been seen in experimental *L. donovani* (Gardener, 1974) and in human *L. tropica major* (Sandbank, 1976) infections.

The survival of the invading parasites in the hamster host cells might be further documented by another of our observations. As far as we know dividing amastigotes have been reported only in macrophages infected *in vitro* (Chang and Dwyer, 1978; Lewis and Peters, 1977) in the form of parasites having two flagella. In the splenic macrophages, but not in human cells, in our material we also found such amastigotes. This finding might also be the result of more favourable conditions for intracellular survival of the amastigotes in hamster macrophages.

In order to deal successfully with the invading parasites the macrophages have to increase their metabolic processes, resulting in the change of several ultrastructural features. A difference was also noted between human and hamster macrophages in this respect. The human macrophages and some hamster cells which contained no or very few amastigotes showed the ultrastructural features of a very active secretory cell. The rough-surfaced endoplasmic reticulum was dilated and filled with a granular material, the Golgi apparatus was well developed and there were many primary lysosomes. Small coated vesicles were seen either close to or fused with the plasma membrane. This latter phenomenon is particularly interesting. Investigations of the past few years have revealed that small coated vesicles (smaller than 90 nm in diameter) are involved in the transport of enzymes, in membrane recycling and in the process of the formation of cellular interconnecting elements (see review by Caputo and Gianotti, 1979). It is also known that infected macrophages have *Leishmania* antigens on their surface (Farah *et al.*, 1975; Handman *et al.*, 1979; Behin *et al.*, 1979). One can speculate that the small coated vesicles found in parasitized human and non-degenerating hamster macrophages might be the morphological elements of the antigen-presenting process.

Finally, it should be pointed out that in our observations the morphological picture of the macrophage-parasite interaction differed to some extent from that seen in *in vitro* experiments. None of these publications included any descriptions of the features of enhanced metabolic activity or coated vesicles, neither was the membranous type of degeneration and rupture of the PV described (Alexander and Vickerman, 1975; Chang and Dwyer, 1976, 1978; Lewis and Peters, 1977; Ardehali *et al.*, 1979; Ebert *et al.*, 1979). The fundamentally different experimental conditions can explain the discrepancies between these and our observations.

The present descriptive work has revealed that the macrophage-parasite interaction shows morphological differences both between humans and hamsters as well as between *in vivo* and *in vitro* conditions. Thus it serves to underline the importance of comparative and *in vivo* investigations for better understanding of the various protective processes in leishmanial infections.

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VISCERAL SPREADING DEPLETION OF THYMUS-DEPENDENT REGIONS AND AMYLOIDOSIS IN MICE AND HAMSTERS INFECTED INTRADERMALLY WITH *LEISHMANIA* ISOLATED FROM SUDANESE CUTANEOUS LEISHMANIASIS

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Summary.—Eighteen outbred mice and 21 golden hamsters were each inoculated intradermally with 2×10^6 *Leishmania* amastigotes obtained from 1 case of Sudanese cutaneous leishmaniasis. The skin lesions, spleen, lymph nodes, liver and kidney were examined by light-, polarizing-, and electron microscope at 5, 9 and 18 weeks after inoculation. The aim of the investigations was to follow the development of the inflammatory reaction and the change of the morphology of the lymphoid organs during the infection.

In all the mice and in the majority of the hamsters visceral leishmaniasis developed which was characterized by a "noncure" type of cellular reaction, a selective T-cell depletion in the lymph nodes and the spleen, and the development of a reactive, systemic amyloidosis. These findings point to the failure of the acquired resistance against *Leishmania* to develop. In some of the hamsters the response was of the "cure" type without the development of amyloidosis.

At the site of the inoculation the lesions healed suggesting the positive role of necrosis and the elimination of the parasites through the ulcer in the healing process.

Electron microscopy showed erythrophagocytosis in the spleen of the 2 mice examined presenting an experimental evidence of the destruction of the red blood cells, which is a common feature of human kala azar.

THE ROLE of genetic control in the course of leishmaniasis in various inbred strains of mice has become known in the past decade, leading to the recognition of resistant and susceptible strains (Bradley, 1974; Preston and Dumonde, 1976; Bradley and Kirkley, 1977; Behin, Mauel and Sordat, 1979; Handman, Ceredig and Mitchell, 1979; Howard, Hale and Chan-Liew, 1980; Rezai, Farrell and Soalsby, 1980; DeTolla, Scott and Farrell, 1981). Recent experiments have also shown that after infection with the "dermatotropic" *L. tropica* or *L. mexicana* visceral leishmaniasis can develop in non-resistant strains of mice (Perez, Labrador and Torrealba, 1979; Leclerc *et al.*, 1981, 1982), which also showed a significant

decrease of T-cells in the spleen (Djoko-Tamnou *et al.*, 1981). These experiments suggest that the development of the various clinical forms of leishmaniasis could be host-dependent.

In the Sudan 3 distinct varieties of human leishmaniasis occur: visceral, mucosal and cutaneous (Sati, 1958; Milosev *et al.*, 1969; Abdalla *et al.*, 1973). Earlier histomorphological investigations of 20 fatal cases of human visceral leishmaniasis revealed a selective depletion of thymus-dependent regions in the spleen and lymph nodes (Veress *et al.*, 1977). Our previous electron-microscopic and morphometric analysis of *Leishmania* amastigotes obtained from all 3 forms of human leishmaniasis from different parts

of the Sudan showed a similar number of subpellicular microtubules, suggesting that these organisms belonged to the same type of *Leishmania*, namely *L. donovani* (Veress, Abdalla and El Hassan, 1980). However, recent investigations showed that *L. major* is the common cause of the cutaneous disease, without excluding the possibility that *L. donovani* is the cause of some cases (Abdalla, 1982). The parasites obtained from human cutaneous lesions produced visceral infection in outbred mice and hamsters (Abdalla, 1982). We thought, therefore, it is worthwhile to investigate the visceral disease in these animals by observing the morphological alterations of the lymphoid system during their infection.

The results of these experiments have shown that the parasites caused visceral involvement with an inflammatory reaction which was of the "noncure" type and it was accompanied by the depletion of the thymus-dependent regions in the spleen and the lymph nodes and reactive, systemic amyloidosis in nearly all of the animals. The different morphological response of the "cure" type seen in some of the hamsters can possibly be explained by genetic differences. In spite of the visceral disease the local skin lesions healed suggesting the role of local factors (necrosis, ulceration) in the healing.

MATERIALS AND METHODS

Parasites.—*Leishmania* amastigotes were isolated from the skin lesion of a patient suffering from cutaneous leishmaniasis by homogenization of a part of the biopsy specimen. This material was injected intradermally into the tip of the nose of golden hamsters. The lesions of the nose were excised and homogenized in Hanks' balanced solution.

Experimental infection.—Eighteen outbred mice weighing about 150 g were infected by intradermal injection of 2×10^6 amastigotes in 0.05 ml Hanks' balanced solution on the back and 21 hamsters weighing about 400 g were given the same dose interdermally into the tip of the nose. The animals were divided into 3 groups (6 mice and 7 hamsters in each group) and killed at 5, 9 and 18 weeks after the inocula-

tion. Five mice and 5 hamsters served as controls having been given only 0.05 ml Hanks' solution intradermally at similar localization and killed at 18 weeks after the inoculation.

Light and electron microscopy.—The skin lesions, visible lymph nodes and part of the spleen, kidney and liver were fixed in 4% formaldehyde in phosphate buffer pH 7.4 rendered isotonic with sucrose for 24 h at 4° and then embedded in paraffin for light microscopy. The sections were stained with haematoxylin and eosin, Giemsa, methylgreen-pyronin and PAS. For polarization microscopy the sections were stained by Congo-red and covered with gum arabic with or without a preceding performiat treatment for the differentiation of primary and secondary amyloid (Romhányi, 1979).

For electron microscopy 1 cm³ cubes of the spleen, kidney and liver from 2 mice and 2 hamsters killed at 18 weeks were fixed in a similar formaldehyde solution at 4° for 4 h. Postfixation was performed in 1% OsO₄ in Millonig's buffer solution at 4° for 1 h before embedding in Durcupan ACM (Fluka AG, Switzerland). Ultrathin sections were cut with an LKB Ultratome III and stained with uranyl acetate and lead citrate. Electron micrographs were taken on Gevaert 23D50 plates with a JEOL 100B electron microscope at the 2nd Central Laboratory for Electron Microscopy, Semmelweis Medical University, Budapest, Hungary.

RESULTS

Skin lesions

A small nodule developed within 1–2 weeks after inoculation at the site of infection both in mice and hamsters. At about 4 weeks the lesions ulcerated. Healing started around 12 weeks and was completed after 16 weeks.

Histology showed at 5 weeks a central large ulcer with fibrin and necrotized cells on the surface (Fig. 1). Beneath this layer there was a thick zone of large, pale, heavily-parasitized histiocytes intermingled with a few plasma cells (Fig. 1, insert). At the periphery there was a dense inflammatory infiltrate dominated by plasma cells (Fig. 2b). In the small arteries within and around the inflammation in 4 mice and 3 hamsters necrosis of the smooth muscle cells or subintimal fibrinoid change could be found (Fig. 2a). At 9 weeks the necrosis in the centre of

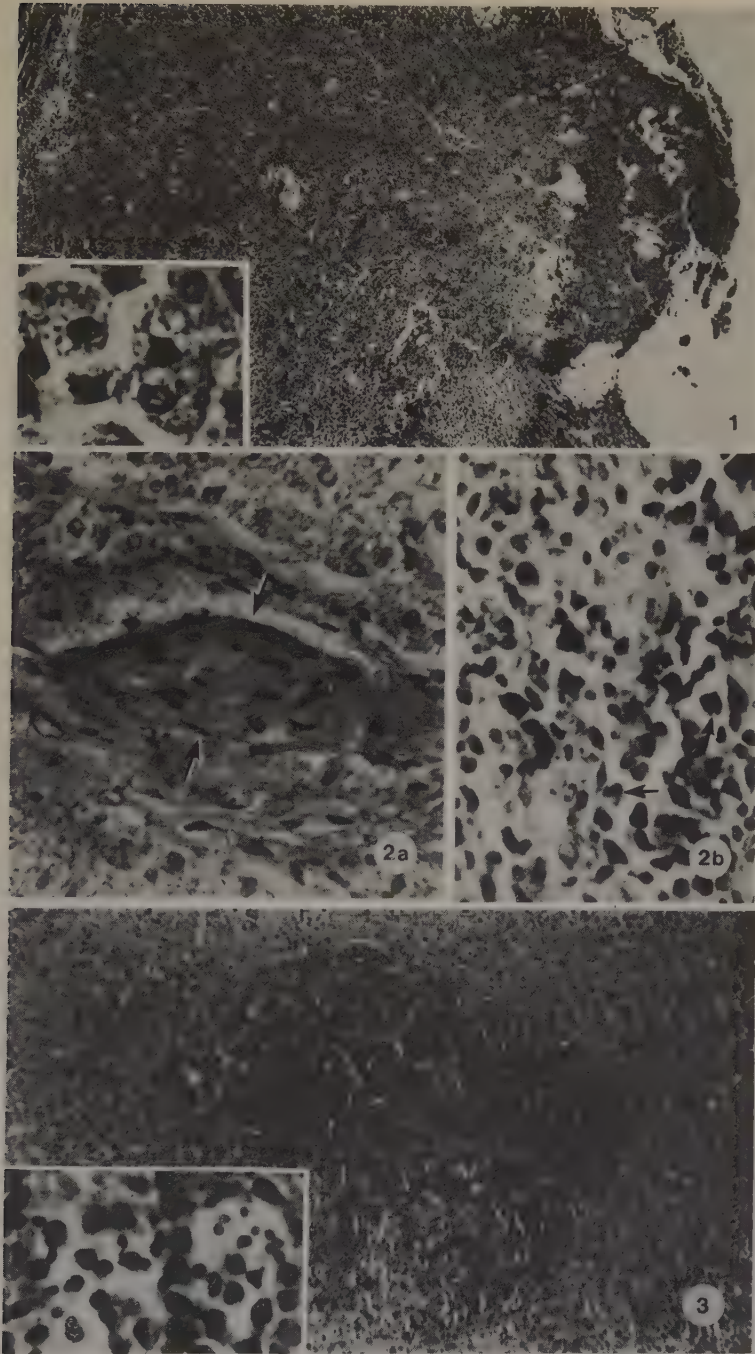


FIG. 1.—Five weeks, mouse, skin. Sharp ulcer with large superficial necrosis. Beneath the necrosis there is a thick layer of heavily parasitized histiocytes. Insert: large histiocytes containing numerous parasites. H. & E. $\times 30$. Insert: $\times 420$.

FIG. 2.—Five weeks, mouse, skin. A: Showing fibrinoid change in a small vessel (arrows). B: Detail of the cell-infiltrate from the periphery with plasma cells (arrows). H. & E. A: $\times 240$. B: $\times 180$.

FIG. 3.—Nine weeks, mouse, spleen. Enlarged Malpighian corpuscle with necrotized lymphocytes. Insert: "tingible body" macrophages containing phagocytosed cell-fragments. H. & E. $\times 60$. Insert: $\times 180$.

the lesion was not so marked, and in the surrounding infiltrate activated, non-parasitized histiocytes with basophilic cytoplasm, accompanied by many small lymphocytes and plasma cells, were seen. Moreover, in 6 hamsters but no mice epithelioid cell granulomata were also observed. 18 weeks after inoculation the lesions were healed by fibrous tissue in all animals.

Lymph nodes

At 5 weeks a marked increase in the number of plasma cells in the medullary cords and the presence of pale, large histiocytes were the most characteristic changes in both mice and hamsters. At 9 weeks the number of small lymphocytes was also increased in the paracortical areas but they were not the dominating cell type in the lymph nodes. In 4 mice and 3 hamsters parasitized histiocytes were present in the subcapsular sinuses. At 18 weeks histology showed many histiocytes, some containing parasites, and a diminished number of small lymphocytes in all mice and 3 hamsters. In 4 hamsters there were no *Leishmania* parasites seen in the histiocytes but the paracortical areas were well populated with small lymphocytes. No amyloid deposits were found in the lymph nodes examined.

Spleen

Splenomegaly was detected in all mice but one, which was killed 5 weeks after inoculation, and in 3, 6 and 5 hamsters killed at 5, 9 and 18 weeks, respectively. At 5 weeks histology revealed enlargement of the Malpighian corpuscles caused by the accumulation of immunoblasts and plasma cells at the periphery and of small lymphocytes around the central artery. In the cords of the red pulp there were also increased numbers of plasma cells and pale, non-parasitized histiocytes. At 9 weeks a striking feature was the necrosis of individual lymphocytes and

the presence of "tingible body" macrophages in the large Malpighian corpuscles (Fig. 3). Perifollicularly, and extending into the red pulp, a homogeneous, eosinophilic material accumulated in all the mice and in 6 of the hamsters (Fig. 4). This material showed green-birefringence after Congo-stain in polarized light, but was isotropic following performic treatment: hence it was "secondary" amyloid. Scattered within the amyloid there were still many plasma cells and histiocytes. Numerous macrophages in all the mice and some of the 6 hamsters contained *Leishmania* parasites. Granulomata of the parasitized histiocytes were also observed in 2 mice and 3 hamsters. The histology of the spleen showed a different picture in one hamster: only scattered necrosis of the lymphocytes and a few "tingible body" macrophages were present in the enlarged Malpighian corpuscles and no amyloid was detected. At 18 weeks the Malpighian corpuscles were dramatically diminished in size and almost completely obliterated due to the accumulation of large masses of amyloid in all mice and in 5 hamsters (Fig. 5). No longer were "tingible body" macrophages seen in the corpuscles, which were composed of a small number of lymphocytes and plasma cells. The dominating cell-type in the amyloid of the red pulp was still the plasma cell along with some pale histiocytes, but the numbers of both were also diminished. In 2 mice and 4 hamsters granulomata composed of parasitized histiocytes were also found with plasma cells at the periphery (Fig. 6). In 2 hamsters neither parasites nor amyloid were seen, and the Malpighian corpuscles were of normal size without signs of necrosis.

Electron microscopy of the spleen revealed 8 nanometer thick filaments typical for amyloid (Fig. 4, insert). In the centre of the granulomata necrotized cells and undamaged amastigotes were observed (Fig. 7). In both mice several macrophages could also be seen containing rests of phagocytosed RBC-s or siderosomes (Fig. 8).

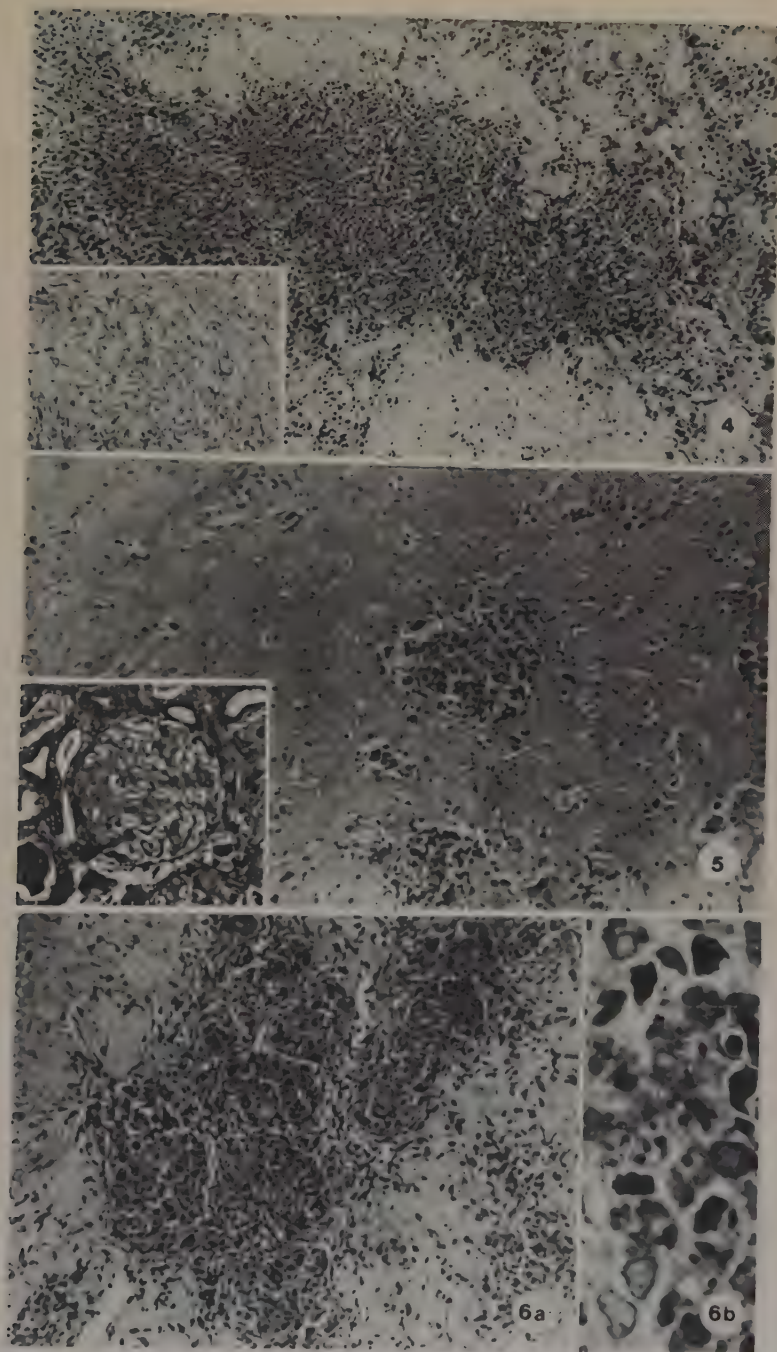


FIG. 4.—Nine weeks, hamster, spleen. Accumulated homogeneous amyloid around the large Malpighian corpuscle. Insert: amyloid filaments of 8 nm diameter. H. & E. $\times 60$. Insert: uranyl acetate and lead citrate. $\times 15,000$.

FIG. 5.—Eighteen weeks, mouse, spleen. Atrophic Malpighian corpuscle and large amount of amyloid with scattered plasma cells. Insert: 18 weeks, hamster, kidney. Amyloid-deposits in the glomerulus. H. & E. $\times 100$.

FIG. 6.—Eighteen weeks, hamster, spleen. A: Showing granulomata surrounded by amyloid. The granulomata are composed of parasitized histiocytes and a few plasma cells at the periphery. H. & E. $\times 140$. B: High power view of parasitized histiocytes from a granuloma. 1 μ m section, toluidine blue. $\times 480$.

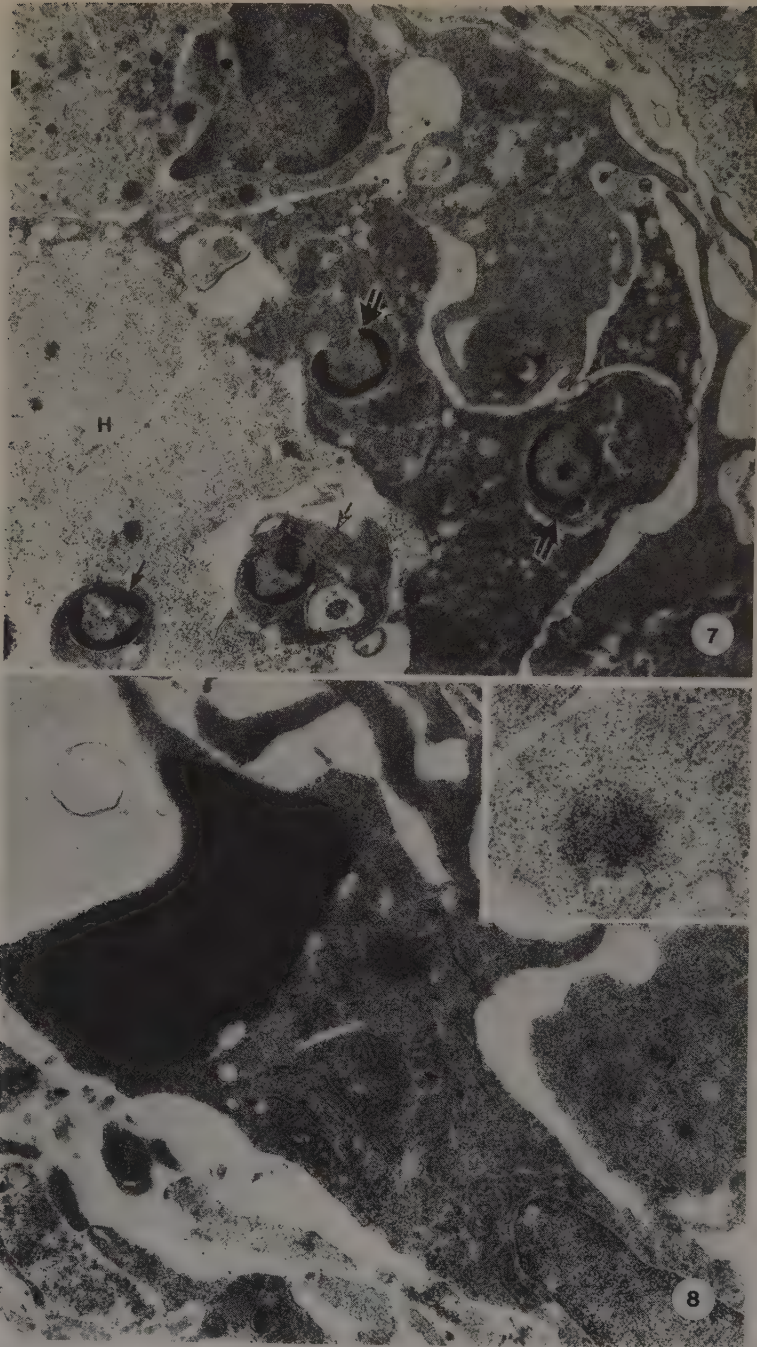


FIG. 7.—Eighteen weeks, hamster, spleen. Detail of a granuloma with remnants of a necrotized histiocyte (H) and viable, extracellular amastigotes (arrows). At the periphery there are macrophages containing amastigotes in the cytoplasm (double arrow). Uranyl acetate and lead citrate. $\times 9600$.
 FIG. 8.—Eighteen weeks, mouse, spleen. Fragment of a phagocytosed red blood cell in the cytoplasm of a macrophage. Insert: siderosome with ferritin granules. Uranyl acetate and lead citrate. $\times 12,000$. Insert: $\times 32,000$.

Kidney and liver

At 5 weeks there was no histological alteration seen in these organs, but at 9 and 18 weeks in all mice and in 6 and 5 hamsters, respectively, the glomeruli and some of the small arteries showed heavy deposits of amyloid (Fig. 5, insert). The liver sinuses were also obliterated in these animals by large amounts of amyloid. Furthermore, in 3 mice and 1 hamster there were granulomata found in the liver composed of parasitized histiocytes.

Control groups

Neither the skin nor the spleen, lymph nodes, kidney or liver showed any pathological changes in the control animals.

DISCUSSION

The defence against infection by *Leishmania* parasites is a complex mechanism, composed of an early, natural, genetically-regulated immunity, and a late, acquired one (Bradley, 1977). A delicate relationship should exist between the cell-types of the natural and the acquired resistances, otherwise the development of the curative cell-mediated immunity will be impaired, and the infection will become a non-healing disease. The natural resistance is under genetic influence (Howard, Hale and Liew, 1982) and the failure of this control is thought to be responsible for the development of a non-healing visceral disease in susceptible inbred mice strains after inoculation with the dermatotropic *L. tropica* (Nasseri and Modabber, 1979; Djoko-Tamnou *et al.*, 1981; Leclerc *et al.*, 1981), while successful control results in a self-healing cutaneous disease in resistant mice strains, even after *L. donovani* infection (Blackwell, Freeman and Bradley, 1980; Blackwell, 1982).

Our experiments seem to strengthen this theory by showing the failure of the acquired resistance to develop in all the mice and in the majority of hamsters. This failure led to the visceral spreading of the parasites, although they were obtained from a human cutaneous lesion.

An impaired acquired immunity was demonstrated in these investigations by a "noncure" type of cellular response, a selective T-cell depletion, and the development of a reactive, systemic amyloidosis while the lack of amyloidosis, the disappearance of *Leishmania* parasites, and the normalization of splenic histology in some of the hamsters can point to the effective role of the genetic control in these animals.

The "non-cure" cellular response

Bradley and Kirkley (1977) described first the occurrence of "noncure" granulomata in outbred PO mice. These granulomata were composed of large, pale, heavily parasitized histiocytes and some plasma cells but no lymphocytes. Similar reaction was also observed in susceptible B10HTG (Blackwell *et al.*, 1980) and in BALB mice (Howard *et al.*, 1980). In our experiments the cellular response was similar with the occurrence of parasitized histiocytes, plasma cells and granulomata of the same type, as well as the scarcity of small lymphocytes and activated histiocytes in the majority of the animals. The cause of the insufficient activation of the macrophages may be either the depletion of T-cells, through the absence of lymphokines from sensitized T-cells (Howard *et al.*, 1982) or their inadequate response due to the failure of the genetic control (Blackwell, 1982).

T-cell depletion

In the present experiments an initial hyperplasia of the Malpighian corpuscles was observed. Later, however, the small lymphocytes necrotized at the centre and "tingible body" macrophages appeared. This necrosis, along with the accumulation of amyloid later on, has led to the atrophy of the Malpighian corpuscles. Parallel with this process the number of small lymphocytes has diminished in the lymph nodes. Similar depletion of T-cells occurred in cases of fatal human visceral leishmaniasis (Veress *et al.*, 1977). These findings are in agree-

ment with the observations of Djoko-Tamnou *et al.* (1981) and Leclerc *et al.* (1982) who found a significant fall in the percentage of T-cells in the spleen of *L. tropica* infected BALB/c mice. The cause of the T-cell depletion is a possible excess-load of antigen. This is known to lead to the induction of specific T-suppressor cells (Gershon and Kondo, 1971).

Amyloidosis

The third sign showing the impairment of the immune response was the development of reactive, systemic amyloidosis, a previously seldom-encountered complication of experimental leishmaniasis. The first histologically documented description of amyloid in hamsters infected with *L. donovani* was published by Gellhorn *et al.* (1946). Amyloid depositions of the spleen, kidney and liver were also observed in *L. mexicana* infected mice and hamsters (Coutinho-Abarth and Coelho, 1965). Duarte, Sesso and Brito (1978) dealt with the role of mesangial cells in amyloidosis of hamsters infected with *L. donovani* without describing the histomorphological changes in other organs. The relationship between impaired cellular immunity and amyloidosis is not as well understood as the connection between the hyperplasia of pyroninophilic cells and amyloid SAA protein-production in the first phase of amyloidosis (Glenner, 1980). It may be that an amyloidogenic factor is released from the necrotizing lymphocytes (Claesson and Hardt, 1972), or the T-cell depletion and amyloid-formation is an expression of immunologic tolerance (Cathcart, Mullarkey and Cohen, 1970). One can also assume that the T-cell depletion caused by antigen overload can lead to a "defect" of the information-exchange between T-lymphocytes and macrophages. As a consequence of this disturbance the macrophages might produce AA fibrils instead of other protein products, hence the development of amyloidosis.

A striking feature of leishmaniasis in our investigations was the discrepancy

between local healing and the development of a systemic disease with impaired cellular immunity. The histomorphological alterations at the site of the parasite inoculation were similar to those described by Ridley (1979) in Old World cutaneous leishmaniasis and by Monroy *et al.* (1980) in *L. enriettii*-infected guinea-pigs. These were an initial infiltrate of pale, parasitized histiocytes, followed by necrosis of these cells and the skin, leading to the ulceration; and in the late phase the accumulation of activated histiocytes and small lymphocytes, resulting in local healing. It was Bryceson *et al.* (1970) who first suggested an immunologically induced necrosis as an important part of the local defence in experimental cutaneous leishmaniasis. Monroy *et al.* (1980) in a detailed study, showed that the majority of the parasites were extruded through the ulcer but that the healing was not a result of the activation of macrophages after the parasite load was reduced. Taking into consideration the various courses of infection locally and in the visceral organs in the present experiments we can assume that it was a local factor (namely, the ulceration) that played a role in the healing. The disturbance of the local circulation can also have been a promoting factor in the necrosis; this is supported by the local vasculitis observed in several animals.

The present investigations also showed ultrastructural evidence of erythrophagocytosis in the spleen of 2 mice. Similar observations were earlier described in cases of human kala-azar (Woodruff, 1973; Veress *et al.*, 1977). Decker-Jackson and Honigberg (1978) have presented data showing that *Leishmania* parasites have antigens cross-reacting with components of blood cells. This could be the basis of autoimmune destruction of the red blood cells leading to haemolytic anaemia, a feature of human visceral leishmaniasis (Woodruff, 1973). In the light of these data one can assume that a similar mechanism takes place in *Leishmania* infected mice.

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